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NITROGENOUS EXCRETORY PRODUCTS OF *TAENIA TAENIAEFORMIS* LARVAE

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The work reported here is a continuation of a study of the catabolic nitrogenous products of parasitic larvae living under axenic conditions. Previous papers have described the results obtained with certain nematode larvae (Weinstein and Haskins, 1955; Haskins and Weinstein, 1957a, b, c). This report extends the observations to another class, the Cestoda.

Apparently there is no published information concerning the nitrogenous products released by cestodes into the ambient medium. However, urea, uric acid, creatinine, and betaine have been found in *Echinococcus* cyst fluid (Mazzocco, 1923; Flössner, 1925; Lemaire and Ribère, 1935; Codounis and Polydorides, 1936); ammonia has been found in the tissues of *Moniezia expansa* (van Grembergen and Pennoit-DeCooman, 1944); Schopfer (1932) reported urea and uric acid from the cyst fluid of *Cysticercus tenuicollis;* and Salisbury and Anderson (1939) found ammonia, urea, and uric acid in the tissue of larval *Taenia taeniaeformis* (*Cysticercus fasciolaris*).

MATERIALS AND METHODS

The *Taenia taeniaeformis* used in the experiments were raised in laboratory rats and mice using naturally infected cats as the egg source. The larvae were taken from the livers 11 to 19 months after the rodents were exposed to infection. At this time the larvae were very large, elongate, and had a very small terminal vesicle. The cysts in which the worms were found were 7 to 9 mm in diameter and the worms ranged in wet weight from 0.10 to 0.63 g.

It was found that the worms could be removed from the infected liver with a minimal risk of contamination. The animal was killed by a blow on the head. The belly was then swabbed thoroughly with tincture of iodine and the animal was completely covered with a sterile cloth which was soaked with tincture of iodine. The abdomen was opened under an operating hood with precautions to avoid contamination. When the body wall and part of the rib cage were removed, revealing the liver, there was ready access to the cysts, most of which were visible on the surface of the liver. The cyst walls were torn open with care to avoid damage to the worms and to the adjacent liver tissue. The worms then were lifted into sterile 0.85% saline solution or into sterile Ringer-Tyrode solution without glucose (Evans, 1956). The worms appeared to react more favorably to the Ringer-Tyrode than to saline. Glucose was omitted from the former solution because it combined with the ammonia excreted by the larvae and thus prevented the determination of ammonia nitrogen in the incubates. The worms were washed through 2 changes of the solution chosen, and, finally, each larva was blotted off and placed singly in a tared 15- by 150-mm tube containing 5 ml of saline or Ringer-Tyrode without glucose. The tubes were tightly stoppered and placed on a roller drum at 37° C for 24 or 48 hours. Following this the worms were removed from the tubes, tested for viability, and discarded. All worms survived the incubation period. Three drops of the incubate from each tube were tested for sterility in thioglycollate medium and in horse meat broth. Occasionally, a tube was found to be contaminated with bacteria or mold and was discarded. Immediately after the incubates were sampled for sterility they were stored at −20° C.

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Chemical analyses

In general the procedures for quantitative and qualitative analyses performed on the incubates were similar to those described for the incubates from nematode larvae Haskins and Weinstein, 1957a, b, c). In some instances modifications were introduced to allow for differences in composition and concentration of the various components and the presence or absence of interfering substances.

Total nitrogen. One-ml samples of the incubates were digested by the Kjeldahl method and the nitrogen determined colorimetrically following nesslerization. The procedure used was essentially that described by Johnson (1941).

Ammonia nitrogen. One-ml samples of the incubate were nesslerized directly for the determination of the ammonium salts. It has been shown that the presence of volatile aliphatic amines does not interfere in this determination (Haskins and Weinstein, 1957a).

Volatile amine nitrogen. One-ml samples were treated with 0.1 ml of 2 N sodium hydroxide and evaporated to dryness *in vacuo* in a desiccator containing concentrated sulfuric acid. The pressure was reduced to 2 to 3 mm for 4 days to remove ammonia and volatile amines. The residues were dissolved in 0.2 ml of water, acidified with 0.1 ml of 6 N sulfuric acid, and the nitrogen content determined by the Kjeldahl method. These results, which represent the non-volatile portion of the total nitrogen, were used to calculate the volatile amine nitrogen content by subtracting the sum of the non-volatile nitrogen and the ammonia nitrogen from the total nitrogen.

Amino acid nitrogen. Because ammonia and volatile amines interfered with this determination, the 1-ml samples were made alkaline with 0.1 ml of 2 N sodium hydroxide and evaporated *in vacuo* as described above. The residues were dissolved in 0.4 ml of water and made slightly acid by the addition of 0.05 ml of 6 N hydrochloric acid. The amino acid nitrogen was then determined by the ninhydrin method described by Snell and Snell (1954a). The standard was 0.2 ml of a solution of 19 common amino acids found in protein hydrolysates. It contained 77 micrograms of nitrogen per ml by Kjeldahl analysis. The blank was 1 ml of Ringer-Tyrode solution carried through the same procedure.

Urea nitrogen. One-ml samples were treated with 1 ml of a solution containing 1 mg of crystalline urease in 1 ml of 1.5 M acetate buffer of pH 6.3. After incubating the samples for 15 minutes at 45° C, they were cooled and the ammonia content measured by nesslerization. At the same time control samples of Ringer-Tyrode solution plus urease reagent, incubate plus buffer without urease, and a urea standard plus urease reagent were treated by the same procedure. The values obtained from the incubate-plus-buffer samples were subtracted from the incubate-plus-urease results to correct for the ammonia originally present in the incubates. The Ringer-Tyrode solution, urease, and buffer contained negligible amounts of ammonia.

Peptide nitrogen. The usual protein and polypeptide precipitants, such as phosphotungstic, perchloric, and trichloroacetic acids, produced no precipitates with the incubates. The biuret reaction gave anomalous results due to formation of haze when the incubates were mixed with the reagent. The differential ultraviolet absorption values of the incubates at 280 and 260 millimicrons also gave impossible results when calculated as protein nitrogen (Kalckar, 1947). However, it was possible to demonstrate that some peptide nitrogen was present by comparison of the color density produced by the Folin-Ciocalteau reagent before and after alkaline hydrolysis of equivalent samples. A small but definite increase in the color density, after hydrolysis, was interpreted as an increase in the amount of free tyrosine present resulting from the cleavage of peptide bonds. It is difficult to estimate the quantitative amount of peptide nitrogen present from these data. An approximation was obtained by assuming that 1 part of tyrosine was equivalent to 11.5 parts of protein as in total protein determinations by this method. Calculation in this way gave peptide nitrogen values which appeared to be of reasonable magnitude.

Creatin and creatinine nitrogen. One-ml samples were examined by the methods C-41a and C-43a described by Fister (1950). No creatin or creatinine was found.

Allantoin nitrogen. One-ml samples were analyzed by the method described by Snell and Snell (1954b). No allantoin was found.

Uric acid nitrogen. One-ml samples were analyzed by the uricase method described by Mahler, Hübscher, and Baum (1955). No uric acid could be detected.

Qualitative analysis of amino acids. The procedure for the identification of the free amino acids in the incubates was essentially the same as that described by Haskins and Weinstein (1957b). A 5-ml aliquot of the incubate was evaporated to dryness *in vacuo*. The residue was dissolved in .1 ml of 0.1 N hydrochloric acid and again evaporated to dryness. After taking up the residue in 0.1 ml of water, the solution was spotted in the center of each of 2 pieces of 24 cm circular Whatman No. 1 filter paper in equal portions. The chromatography was carried out as described by Oreskes and Saifer (1955). Identification of the amino acids was by comparison with chromatograms prepared from mixtures of known amino acids and by Rf values.

Qualitative analysis of amines. A 10-ml aliquot of incubates was acidified with 0.1 ml of 0.1 N hydrochloric acid and evaporated to dryness *in vacuo*. The residue was extracted with three 0.5-ml portions of absolute alcohol. The combined extracts were acidified with 0.05 ml of 10% alcoholic hydrochloric acid and evaporated to dryness in a desiccator. The residue was dissolved in 0.1 ml of absolute alcohol and 40-microliter aliquots were spotted on Whatman No. 1 filter paper sheets for chromatographic identification of the amines as described by Weinstein and Haskins (1955) and Haskins and Weinstein (1957c). A 10-ml sample of Ringer-Tyrode solution was carried through the same procedure as a control. No contaminating substances were detected.

RESULTS

A total of 10 pools of incubates was examined; each pool contained the incubates from 4 to 12 larvae. A total of 76 larvae, averaging 0.28 g wet weight, was used in the study. The results of the quantitative analyses were calculated as micrograms of nitrogen per g of larval wet weight. The yield was 400 to 600 micrograms per g on this basis. The incubation period was 24 hours for 6 sets of incubates and 48 hours for the remaining 4 sets of incubates. The added period of incubation did not add significantly to the amount of nitrogen per g recovered and the data obtained from the 2 sets of experiments are not separated in the results reported.

TABLE I.—*Type and amount of nitrogen excreted by* T. taeniaeformis *and* T. spiralis *larvae.*

	T. taeniaformis		*T. spiralis*	
	Average (%)	Range (%)	Average (%)	Range (%)
Ammonia	17.5	14–20	33.3	28–37
Volatile amine	15.0	10–20	7.4	2–12
Urea	33.0	26–43	0.0	—
Amino acid	11.7	5–20	28.5	25–32
Peptide	27.0	25–30	20.8	15–28
Total	104.2		90.0	

Table I gives the results obtained from the quantitative analyses in terms of percentage of total nitrogen. In the first column are the average values and in the second the maximum and minimum figures. The averages add to slightly more than 100%. This figure is well within the experimental error of the various determinations, especially considering the value for peptide nitrogen which, as explained under *Methods,* is an approximation. Similar data previously obtained for *Trichinella spiralis* are included in the table for purposes of comparison (Haskins and Weinstein, 1957a).

The qualitative analyses of the volatile amine fractions showed the presence of ethylene diamine, cadaverine, 1-amino-2-propanol, methyl amine, ethyl amine, and butyl amine. A substance which gave a spot with an Rf value of 0.17 with butanol-acetic acid and 0.49 with phenol was observed. This appears to correspond to the same unknown substance which was present in the incubates from *T. spiralis* and *A. lumbricoides larvae* (Haskins and Weinstein, 1957c).

The free amino acids in the incubates were identified as glycine, alanine, valine, methionine, leucine, proline and tyrosine. A band having a low Rf value with both butanol (0.15) and phenol (0.13) was similar to one of the unidentified bands appearing among the amino acid constituents in *T. spiralis* larvae incubates (Haskins and Weinstein, 1957b). It was suspected that this band might represent a polypeptide constituent. To test this, 5-ml aliquots of 2 different incubates were evaporated as described under *Methods* for the qualitative amino acid determination. The dry residues were dissolved in 0.5 ml of 6 N hydrochloric acid and heated in a sealed tube at 100° C for 24 hours. The hydrolysates were evaporated to dryness, taken

up in 0.1 ml of water, and the amino acids identified by circular paper chromatography as before. The band under suspicion was still present and hence could not be due to a polypeptide. However, this experiment did offer additional proof that polypeptides were present in the unhydrolyzed incubate because the bands corresponding to the amino acids mentioned were intensified and 1 new band indicating the presence of glutamic acid appeared.

DISCUSSION

It may be assumed that all the nitrogenous material recovered in the medium, following incubation with the worms, was a product of catabolic activity of the worms. Since the worms have no digestive tract, there could be no contribution to the medium of digested or partly digested food. Also, the worms have no known glands which might be expected to secrete nitrogen-containing substances into the medium.

Presumably, the nitrogenous compounds reaching the medium came through the thin, non-cellular cuticle but there is a possibility that some of the material recovered may have reached the medium in fluid released from the so-called excretory system. In *T. taeniaeformis*, the major "collecting" tubules run longitudinally in the strobila, join posteriorly, and open to the outside through a single pore on the terminal bladder. In addition, there may be accessory openings from the excretory tubes in the strobila (Bartels, 1902). Although an excretory function has been attributed to this system, its actual function is unknown. Wardle and McLeod (1952) call it the "osmoregulatory system" and consider that it may function primarily to regulate hydrostatic pressure in the worm.

Qualitatively the nitrogenous compounds in the incubates of *T. taeniaeformis* larvae bear a close resemblance to those present in incubates of *T. spiralis* larvae. The notable difference is the appearance of 1/3 of the total nitrogen in the incubates of *T. taeniaeformis* larvae as urea. None of this substance was detected in the incubates of the nematode larvae (Haskins and Weinstein, 1957a).

Quantitatively it is apparent that the rate of production of nitrogenous material by the *T. taeniaeformis* larvae is much lower than that of the nematode larvae. By comparison, 1 g wet weight of *T. taeniaeformis* larvae producing 500 micrograms of nitrogen in 24 hours is equivalent to 35 mg wet weight of *T. spiralis* larvae producing at the same rate. Thus, the nematode larva is, under comparable conditions, about 28 times as active metabolically as the tapeworm larva.

The absence of precipitable protein in the incubates may have been due to the absence of glandular secretions or discharge from a digestive tract. The peptides encountered in the incubates were of relatively low molecular weight since they were not precipitated by phosphotungstic acid. The source for these peptides is not known but there appears to be no reason to doubt that they could have passed through the cuticle or could have been released from the excretory system.

SUMMARY

T. taeniaeformis larvae were incubated aerobically under axenic conditions. Chemical analyses of the incubates showed the presence of 400 to 600 micrograms of nitrogen per gram of larvae, net weight, for the first 24 hours. Incubation for 48 hours produced no significant increase in the amount of nitrogenous materials recovered per g wet weight of larvae. The products consisted of 17.5% ammonia

nitrogen, 15% volatile amine nitrogen, 33% urea nitrogen, 11.7% amino acid nitrogen, and 27% peptide nitrogen. Qualitative analysis of the volatile amines disclosed the presence of ethylene diamine, cadaverine, 1-amino-2-propanol, methyl amine, ethyl amine, and butyl amine. A similar analysis of the free amino acid fraction showed the presence of glycine, alanine, valine, methionine, leucine, proline and tyrosine.

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STUDIES ON *HYDATIGERA TAENIAEFORMIS*
I. GROWTH OF THE LARVAL STAGE

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In recent years valuable contributions have been made to cestode physiology by the employment of in vitro cultivation techniques. Workers in this field have mostly used the plerocercoids of the pseudophyllidean worms *Schistocephalus, Ligula* and *Diphyllobothrium* (Smyth, 1955, 1958).

Research on the in vitro cultivation of cyclophyllidean cestodes has not progressed since the work of Wilmoth (1945) who maintained the larvae of the cat tapeworm, *Hydatigera taeniaeformis*, in vitro in order to study cestode respiration. This tapeworm is suitable for cultivation work for several reasons. Firstly, the larval form or strobilocercus exists aseptically within a capsule embedded in the livers of infected rodents and can be transferred easily to culture media without bacterial contamination. Secondly, the larva is already strobilated and the only apparent difference from the adult is the absence of genitalia. Thus somatic growth may not be an essential precursor to the elaboration of genitalia as is the case if cysticerci of *Taenia* spp. are employed. Thirdly, it can be maintained with ease in small laboratory animals.

Although the outline of the life history of *H. taeniaeformis* is well known, no detailed studies have been made of its life and growth. The present paper results from a study of the growth of the larval worms in the liver of mice. This knowledge of larval growth is a prerequisite of in vitro cultivation because it is essential to know at what stage worms become infective if it is hoped to induce maturation in vitro.

MATERIALS AND METHODS

The mice which were infected were offspring of an inbred strain known as Strain A obtained originally from the Imperial Cancer Research Fund, London. It had been found previously that they were highly susceptible to infection with *H. taeniaeformis*. Only mice between the ages of 45–75 days were used as they were refractory to infection outside these limits. (*c.f.* age resistance to infection with larval *H. taeniaeformis* shown by albino rats, Greenfield 1942). Measured amounts of *H. taeniaeformis* eggs suspended in water were administered by stomach tube. The latter consisted of a No. 17 hypodermic needle with the tip covered by a short length of narrow bore (0.5 mm) polythene tubing.

Eggs were obtained from the gravid proglottids of adult *H. taeniaeformis* which had been recovered from the intestine of cats. After washing these worms in tap water, the posterior 6 proglottids were removed and the stage of development of the contained embryos checked. If the eggs were found to be fully embryonated, the proglottids were stored in balanced saline at 4°C. Frequent changes of saline were necessary due to bacterial growth. Material stored in this manner for 5 months was found still to be infective.

Usually, the gravid proglottids were used within a few days of removal from the cat. The proglottids were halved longitudinally along the main axis of the uterus and the eggs pressed out of the lateral branches. An aqueous suspension was then prepared for administration to mice. As the eggs settle rapidly in water, the suspension was shaken thoroughly before being drawn into the syringe. A bubble of air was also drawn in, which facilitated mixing within the syringe between administrations to each mouse.

Proglottids stored for longer than 2 weeks before use tended to degenerate and become brittle. After this change had occurred, the eggs were not readily released and the proglottids

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had to be homogenised at low speeds. The released eggs were unaffected by this treatment but the proglottids disintegrated and the resulting fragments were small enough to pass through the stomach tube without causing blockage.

The growth rate was estimated by killing the mice at varying intervals after infection and comparing the weight of the larvae which had been removed and dried for 24 hours at 105°C. Although large strobilocerci can be surface-dried satisfactorily by blotting in order to obtain the fresh weight, it becomes progressively more difficult to surface-dry the smaller specimens and weigh with accuracy. Estimations of growth were based, therefore, on increase in dry weight.

RESULTS

1. *Morphological Development*

In order to determine the stage of development, the larvae were removed from their host capsules at intervals after infection. The cysts were clearly visible to the naked eye, being 0.5 m in diameter, at the end of the 7th day. The contained larvae were in the form of undifferentiated bladders. By the end of the 14th day, the cysts were 2 mm in diameter and a cephalic rudiment had developed by an inpushing of the bladder (Figs. 5 & 6).

Larvae examined on the 30th day were found to have developed into invaginated cysticerci complete with suckers and hooks (Fig. 7). Too few larvae were obtained from light infections to be accurately weighed, but 200 larvae at the same stage of development were obtained from heavily infected mice. From these it was found that the average dry weight of the invaginated cysticercus was 0.87 mg. Feeding experiments indicated that worms at this stage of development were not infective to cats.

On the 42nd day a precocious evagination of the complete cysticercus was observed (Figs. 8 & 9). Strobilated larvae were recovered on the 48th day (Fig. 10). The weight and age at which they became infective to the definitive host was determined by feeding a series of larvae to cats. The results showed that larvae had to undergo a minimum period of 60 days in the mouse before becoming infective. The condition and appearance of a 62 day infective strobilocercus is shown in Figure 11. The average fresh weight of such larvae is 20 mg.

Examination of the complete strobilae of 169 strobilocerci failed to reveal any trace of genital anlagen. This was confirmed by sectioning 7 individuals.

2. *Degeneration of Larvae during Development*

During the course of infection in the mouse, it was frequently observed that when hexacanths were established in the liver, degeneration occurred within the first 14 days and the cysts were replaced by small yellow scars. Sometimes well developed strobilocerci which had been in the liver for periods up to 4 months, were dead on removal from the cyst. Bullock and Curtis (1924) report that the death of the older larvae is followed by atrophy of the cyst and sometimes by complete replacement fibrosis.

Although the cysts and their contents are normally aseptic, bacteria sometimes gain entry. There is a consequent suppuration of the cyst accompanied by death and degeneration of the contained larva. Bacterial examination of such cysts from mice revealed a Gram-positive pleiomorphic bacillus, *Corynebacterium murium*.

The host capsule is normally transparent and the presence of the strobilocercus gives the cyst a white appearance. Black cysts are frequently encountered in the heavier infections exceeding 6 months' duration. This abnormal color is due to the fact that an internal hemorrhage has occurred, flooding the cyst with blood. The

contained strobilocerci are distinctly yellow in color but are morphologically normal and capable of infecting cats.

3. *Growth of Larva*

Figure 1 shows the growth rate of larval *H. taeniaeformis* in the mouse liver.

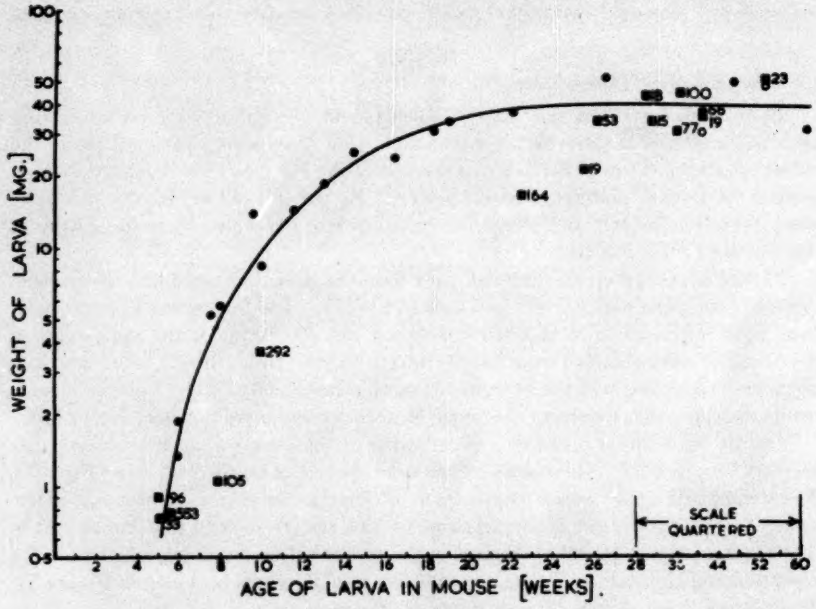

FIGURE 1. Growth of larval *H. taeniaeformis* is plotted on a logarithmic scale and shows the increase in dry weight at successive intervals of time.

■ = The average dry weight of worms from one heavily infected mouse. The index represents the worm burden.

● = The average dry weight of light worm burdens (1–12 worms/mouse). Result based on worms recovered from several mice.

○ = The average dry weight of a light worm burden recovered from a single mouse.

The ordinate represents the age of the larva in the mouse, and the abscissa, the dry weights of the larval cestodes. Since strobilocerci develop from identical cellular masses, and, since the stage at which growth commences is the same in each case, weight can be employed as a direct measure of growth. No estimations of the dry weight were made earlier than 35 days because the larvae were so small that the removal of the large numbers required for an accurate weight was impracticable.

Points represent the mean dry weight of cestodes from lightly infected mice bearing not more than 12 cysts per liver. Although up to 12 worms per mouse was considered as a light infection, 66% of the points were obtained from mice bearing less than 6 worms. A closed point indicates that the estimation is based upon material recovered from several mice while an open point indicates that only a single mouse was examined.

The mean dry weight of cestodes recovered from heavy infections in single mice are plotted individually and are shown on the Figure as solid squares. In each

case the total number of cestodes present in the liver of the heavily infected mouse is recorded as an index to the right of the square. Heavily infected mice were easily distinguished from lightly infected ones due to the tremendous distension of the abdomen (Figs. 2 & 3). One mouse, having been infected for 36 weeks, weighed 66.9 gm and contained 100 worms in the liver. The fresh weight of the worms when removed was 24.4% of the weight of the mouse. This is by no means an exceptional case as involvement of the liver to this extent was common. The condition of the liver in one particular mouse, carrying a burden of 233 worms, is shown in Figure 4.

4. *The Weight of the Oncosphere*

The oncosphere is the stage at which growth commences in the mouse liver. If the dry weight could be estimated and compared with the weight of larva recovered after 6 weeks, it would give an indication of the rate of growth during this early phase of larval development. Direct estimations cannot be made on the weight of the oncosphere but an approximate dry weight in grams can be obtained by multiplying the volume of the oncosphere in cc by the specific gravity (s) and the dry/fresh weight ratio (w).

Since the oncosphere can be seen through the embryophore, it can be measured. Some are ovoid having a length of 21 microns and a breadth of 18 microns while others are spherical with a diameter of 20 microns. From this latter measurement, the volume can be most easily obtained.

The eggs of *H. taeniaeformis*, being dense, settled rapidly in a solution having a specific gravity of 1.19. When the embryophores were removed with hatching fluid (Silverman, 1954) and the oncospheres placed in a graded series of NaCl solutions, it was observed that they sank in a solution of specific gravity 1.04 and floated in another of specific gravity 1.07. The specific gravity of the oncospheres lies between these 2 limits and was taken as being 1.055.

The dry/fresh weight ratio of the oncosphere of any tapeworm is unknown but in the following calculation its value is taken as being 0.27 which is equivalent to the average dry/fresh weight ratio of the larval worm in the liver of the mouse.

The dry weight of the oncosphere is, therefore,

$$4/3\pi r^3.w.s. = 1.33 \times 3.14 \times (10^{-2})^3 \times 0.27 \times 1.055$$
$$= 1.193 \times 10^{-6} \text{ mg i. e. } 1.193 \text{ millimicrograms}$$

Even allowing for an error of ± 50% in the dry/fresh weight ratio, the weight of the oncosphere lies in the range 0.6–1.8 millimicrograms.

DISCUSSION

The weight of an oncosphere, when it becomes established in a mouse liver, is approximately 1 millimicrogram. This weight increases 1 million times during the first 40 days resulting in a larva of 1 mg (Fig. 1). As no intervening figures have been obtained during this period, the shape of the growth curve is not shown. After the 40th day, the rate of growth (by which is meant the length of time taken to double the dry weight) decreases rapidly, and by the 22nd week has virtually stopped. This conclusion is different from that of Sweatman & Plummer (1957) who found that growth in *Taenia hydatigena*, developing in a lamb, was constant throughout the period of investigation (9 months). This comparison, however, is not necessarily valid as these workers measured growth as increase in "linear dimension" of the cyst. Whether the scolex and neck region, or even the total dry weight of the whole larva, continued to increase was not determined.

At least 2 physiological reasons can be advanced to explain the slowing and ultimate cessation of growth of *H. taeniaeformis* occurring in light infections. The first is that the host capsule, which forms around the larva, gradually limits the rate of entry of metabolites. Stoner & Hankes (1955) have shown that this occurs during the development of *Trichinella spiralis* larvae. Secondly, Lewert & Lee (1956) who investigated the physiological processes taking place during the early growth phase of larval *H. taeniaeformis* by means of histochemical techniques, have suggested that the production of a collagenase-like enzyme, which reaches a maximum in the early larva, facilitates the rapid absorption of nutrients by softening the intercellular material and basement membranes of the liver cells. The decreased production of this enzyme by older larvae may in turn lead to a decrease in available food.

While either or both of these processes may be contributory factors, it seems probable that this slowing of growth is not just a chance result of starvation due to a thick host cyst or low collagenase production. If it were, a much greater variation in the size of larvae would be expected. The smoothness in the fall-off in the rate of growth (Fig. 1) suggests a more precise control mechanism. Although there is no direct evidence of such an intrinsic control mechanism, the similarity between this growth curve and that of many other animals suggests that growth is being limited as a result of genetically controlled changes in hormone concentration rather than as a pathological response to extrinsic factors.

A considerable population of larvae can be supported by a single mouse (Fig. 1) but individual mice bearing more than 200 larvae were never observed beyond the 11th week. There is little difference in the amount of growth which has taken place in the widely differing worm burdens of 553 and 33 worms plotted between the 5th and 6th weeks. This shows that competition for available growth substances is absent at this stage. Moreover, both these results lie on the graph of the light infections, and so, during the initial rapid growth phase, the growth rate is constant and is not affected by the worm burden. Beyond the 6th week, it is evident that some limiting factor is depressing the growth rates of the heavier infections. No permanent stunting of the worms was observed and no cases were recorded in which the average maximum size was not reached by the 26th week after infection.

Conflicting reports have appeared as to whether or not there is a genital rudiment present in the proglottids of the strobilocercus of *H. taeniaeformis*. Nelson (1924) reported the presence of an anlage in the larvae of *H. taeniaeformis* recovered from a musk rat. Rees (1952) in her extensive investigations on the anatomy of the strobilocercus, did not observe any trace of an anlage in 9 individuals from 8 rats, a result confirmed in the present work. Rees suggested that its presence may be a variable feature but it is extremely doubtful whether the structures described by Nelson (1924) were, in fact, genital rudiments. In his paper, photographs are shown of the "rudiments" which do not in any way resemble the true genital rudiments making their first appearance in the cestode after 4 days within the definitive host.

A mature strobilocercus such as shown in Figure 8, is remarkably similar in outward appearance to the adult *H. taeniaeformis*. It was this superficial resemblance which gave the early research workers, Von Siebold, Thompson, and Kuchenmeister, the idea that *Cysticercus fasciolaris* in the liver of small rodents was, in fact, the juvenile form of *Hydatigera taeniaeformis* found in the small intestine of the cat. As a result, the relationships of the other cysticerci with the taeniae became evident.

The infective strobilocercus of *H. taeniaeformis* consists of 2 distinct portions. In the anterior, there is a robust and highly contracted region called the *true larval strobila* which survives digestion in the cat. The remainder of the worm, which is digested, is a flimsy and relaxed region called the *pseudostrobila*. The exact demarcation of these 2 portions can not be determined by external examination. Internally, according to Joyeux & Baer (1938) differences in the musculature exist and there are less calcareous corpuscles in the pseudostrobila. It was the opinion of Leuckart (1878) that the complete larval strobila, with the exception of the neck and scolex, was digested away and that the growth of the adult commenced from this point. Miller (1932) disproved this and, by counting the proglottids of the strobilocercus in order to compare them with the number observed after a period of 24 hours in the intestine of the cat, he was able to show that only 25% of the proglottids had been digested. A more detailed account of the relationship between strobila and pseudostrobila is in preparation.

Finally, from the information presented in this paper, a conclusion can be drawn as to the most suitable size of strobilocercus for cultivation. Mendelsohn (1935), who was the first to attempt cultivation of larval *H. taeniaeformis* aseptically, selected the undifferentiated bladder which he removed from rat liver 15 days after infection. After culturing in roller tubes for 35 days, he observed that the cephalic rudiment had developed. Successful cultivation of this stage will give information concerning the metabolism of the larval worm. If, however, the object is to obtain in vitro the development of the adult worm, then it is apparent that the larva should be at least 60 days old, as this is the minimum age of larva capable of maturing in vivo. This applies only to larvae from light infections as growth in heavy infections is retarded and the larvae take longer to reach the infective size. Therefore, a better criterion than age is fresh weight, the critical size being about 20 mg.

SUMMARY

Investigations on the development and growth of larval *Hydatigera taeniaeformis* in Strain A mice have shown that, on the 30th day after infection, an invaginated cysticercus developed. This evaginated on the 42nd day, and by the 48th day a strobilocercus had formed. The youngest strobilocerci capable of infecting cats had a fresh weight of 20 mg and were recovered from mice after 60 days. Rapid growth occurred during the first 6 weeks. Thereafter, the growth rate slowed down and, with light worm burdens, growth stopped 22 weeks after infection. Heavy and light worm burdens grew at the same rate initially, but, beyond the 6th week, the growth rate of worms in heavy burdens was retarded.

ACKNOWLEDGMENTS

I am extremely grateful to Dr. C. A. Hopkins for his advice and criticism of this paper. I am also indebted to the Director of the Imperial Cancer Research Fund for making the original Strain A mice available and to Dr. P. H. Silverman for supplying the hatching fluid for the *Hydatigera* ova. My thanks are also due to Professor C. M. Yonge, G.U.Z.D., and Professor J. P. Todd, R.C.S.T., Glasgow, in whose departments this work was performed.

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Explanation of Plates

FIGURE 2. Control (left) and experimental male mice 87 days after infection.

FIGURE 3. The same mice opened to show the numerous strobilocerci established in the liver.

FIGURE 4. The complete liver of another laboratory infected mouse from which 233 larvae were recovered. Several contracted strobilocerci which have been removed from their host capsules are shown below the infected liver. The scale is in cm.

FIGURE 5. Larva removed from mouse liver 14 days after infection consisting of a simple bladder (b) and inverted cephalic rudiment (c.r.).

FIGURE 6. The bladder (b) of the same larva ruptured to show the cephalic rudiment (c.r.).

FIGURE 7. The "invaginated cysticercus" stage 30 days after infection of the mouse showing bladder (b) invaginated scolex (s) and neck (n).

FIGURE 8. The invaginated cysticercus undergoing a precocious evagination on the 41st day.

FIGURE 9. An "evaginated cysticercus" on the 42nd day.

FIGURE 10. A young strobilocercus after 48 days of development in mouse liver. The strobilated region (str) can be seen to the posterior.

FIGURE 11. A mature strobilocercus 62 days after infection of the mouse. Infective to cats.

PLATE I

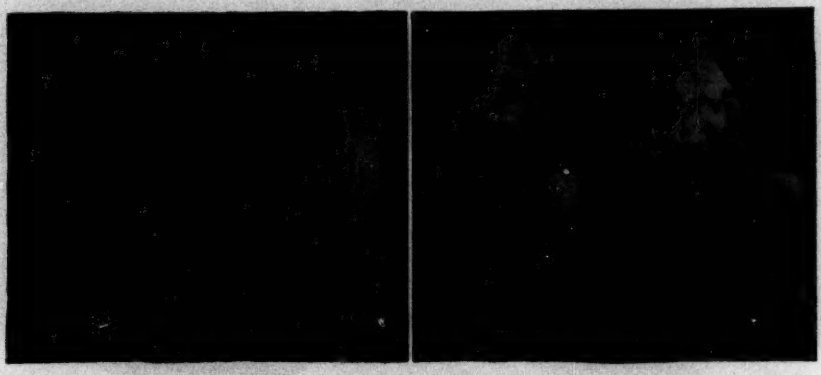

FIG. 2. FIG. 3.

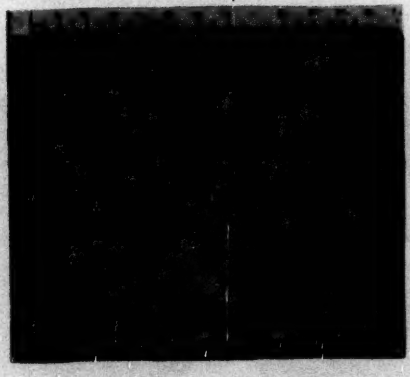

FIG. 4.

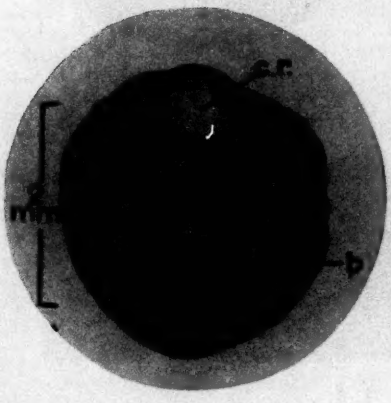

FIG. 5.

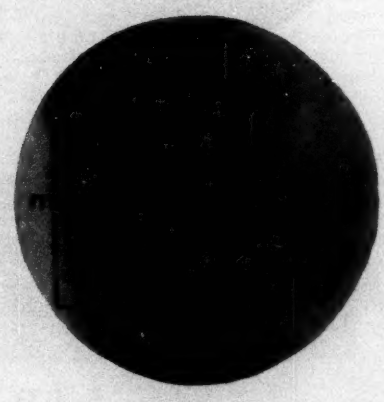

FIG. 6.

PLATE II

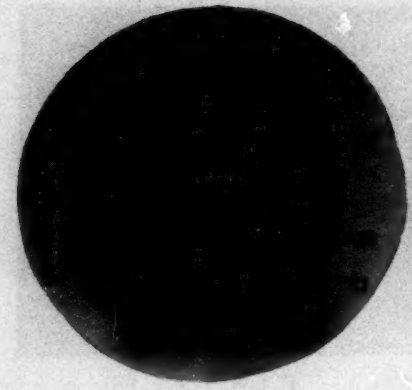

FIG. 7.

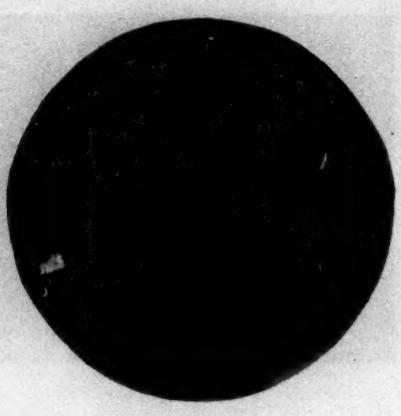

FIG. 8.

FIG. 9.

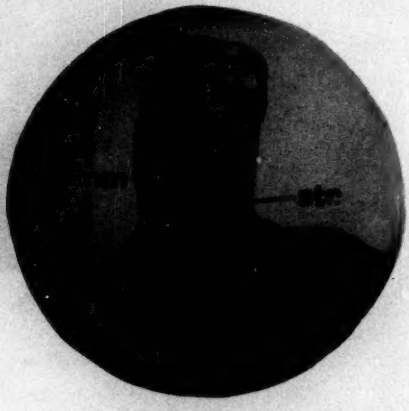

FIG. 10.

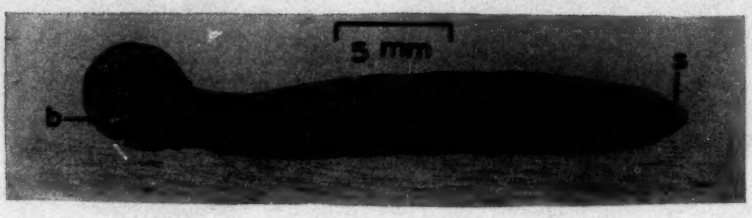

FIG. 11.

OCCURRENCE OF A *TRYPANOSOMA CRUZI*-LIKE ORGANISM IN SOME MAMMALS FROM SOUTHWESTERN GEORGIA AND NORTHWESTERN FLORIDA

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Report of the occurrence of American trypanosomiasis (Chagas' disease) in the United States (Woody and Woody, 1955) has alerted workers in the field to the possibility that the disease may occur elsewhere in the southern United States. The etiologic agent of the disease, *Trypanosoma cruzi*, is known to have been present in this country since 1933 when it was isolated from *Triatoma protracta* by Kofoid and Donat (Wood and Wood, 1941). Since then, it has been found in other *Triatoma* spp. and in mammalian hosts as well. The following mammals have been reported as hosts of *T. cruzi* in the United States: armadillo, opossum, pallid bat, house mouse, 3 species of wood rats (Usinger, 1944), and the raccoon (Walton et al, 1956). In the present study, a *T. cruzi*-like organism was detected in wild mammals from southwestern Georgia and northwestern Florida while studying the epizootiology of leptospirosis in that area. This paper reports the isolation of the organism from 107 animals representing 4 species.

METHODS

The techniques used (McKeever et al, 1958) were devised for investigations on leptospirosis, and are not necessarily recommended for the study of trypanosomiasis specifically. A total of 1,974 animals was trapped by standard procedure, brought to the laboratory alive, bled by cardiac puncture, and killed by injecting air into the blood stream. The kidneys were removed aseptically, and 1 kidney or a portion of 1 kidney from each animal was ground in sufficient isotonic saline, adjusted to pH 7.2–7.4 with sodium phosphate, to make a 10–15% suspension by weight. Three drops of the suspension were used to inoculate 5 ml of each of 4 types of leptospiral media in screw-cap culture tubes. The following media were used: Chang's semi-solid medium (Reinhard, 1953) with 10% rabbit serum, Chang's semi-solid medium with 15% horse plasma, Fletcher's semi-solid medium (Fletcher, 1928) with 10% rabbit serum, and Fletcher's semi-solid medium with 15% horse plasma. All media were buffered to pH 7.2–7.4 and were tested for sterility before inoculation. After inoculation, cultures were incubated for 30 days at 28° C and then examined by darkfield microscopy. The type of media in which the trypanosomes were detected was recorded. Contaminated cultures were not considered an adequate test. In addition to the trypanosomes detected in primary cultures, those detected in subcultures of leptospirae also were recorded.

Equal volumes of both urine and kidney tissue suspension from 27 opossums were tested for leptospirae. Urine was removed from the bladder aseptically, and 3 drops were used to inoculate each of 2 tubes of Chang's and 2 tubes of Fletcher's semi-solid media containing 15% horse plasma. The cultures were treated in the same manner as the cultures of kidney tissue.

After observing trypanosomes in leptospiral media, specific media were used for examination of material collected subsequently. Blood from the heart of 149 opossums was used to inoculate Offutt's (1946) diphasic medium for hemoflagellates. Small amounts of penicillin and streptomycin sulphate were added to the medium to control contamination. One tube of medium was used for each animal. The cultures were incubated for 5 to 8 days at 28° C and then shipped

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to the Laboratory Branch, Communicable Disease Center, where they were examined for trypanosomes. Kidney tissues from 119 of the 149 opossums also were cultured.

Isolates from kidney tissue of 5 opossums, 1 striped skunk, and 1 raccoon were studied in culture, in triatomine bugs, and in mouse blood and tissue by the CDC Laboratory Branch. The Corpus Christi strain and the Brazil strain of *T. cruzi*, and a strain of *T. rangeli* were used for comparison.

RESULTS

From November 30, 1954 to April 19, 1957, wild mammals were trapped on 22 areas in 9 counties of southwestern Georgia and 1 county of northwestern Florida. Trypanosomes were detected in primary cultures for leptospirae from 98 of 1,974 animals representing 14 species. Host species and prevalences of infection were as follows: 88 of 552 opossums, 8 of 608 raccoons, and 2 of 306 striped skunks (Table I). In addition, 1 opossum, 1 raccoon, 1 striped skunk, and 2 gray foxes

TABLE I.—*Number of isolates of* Trypanosoma cruzi*-like organisms obtained from cultures inoculated with kidney tissue from 14 species of mammals from southwestern Georgia and northwestern Florida.*

Species	Positive			Percent Positive
	Number Examined	Primary Culture	Subculture*	
Opossum *Didelphis marsupialis*	552	88	1	16
Cottontail rabbit *Sylvilagus floridanus*	203			
Marsh rabbit *Sylvilagus palustris*	4			
Gray squirrel *Sciurus carolinensis*	3			
Fox squirrel *Sciurus niger*	48			
Red fox *Vulpes fulva*	18			
Gray fox *Urocyon cinereoargenteus*	118		2	1.7
Feral dog *Canis familiaris*	1			
Raccoon *Procyon lotor*	608	8	1	1.5
Striped skunk *Mephitis mephitis*	306	2	1	1.0
Spotted skunk *Spilogale putorius*	7			
Otter *Lutra canadensis*	2			
Wildcat *Felis rufa*	63			
Feral house cat *Felis domestica*	41			
Total	1,974	98	5	5.2

* Duplicates of primary culture not included.

were found to be infected by subcultures made from approximately 200 cultures containing leptospirae. Thus it is probable that a number of positive animals were not detected, since subcultures were made from only about 10% of the animals tested.

Cultures were inoculated with both urine and kidney tissues from 27 opossums (Table II). Viable trypanosomes were obtained from the urine of 2 of the opos-

TABLE II.—*Comparison of methods by which trypanosomes were isolated from opossums collected in southwestern Georgia and northwestern Florida.*

Culture Inoculated With	Numbered Examined	Number Positive	Percent Positive
Kidney	552	89*	16
Urine	27	2	7.4
Blood	149	9	6.0

* 88 from primary culture and 1 from subculture.

sums. The kidney culture of one was positive and that from the other was contaminated. Six of the animals had positive kidney cultures and negative urine cultures.

Tubes of hemoflagellate media were inoculated with blood from 149 opossums and 9 were positive. Cultures were inoculated with both blood and kidney tissues from 119 of these animals. Of these, 13 were positive by kidney culture, 7 were positive by blood culture, and 6 were positive by both tests. A comparison of the differences in percentage of infection as determined by cultures inoculated with kidney tissue, urine, and blood (Table II) revealed that only the difference between kidney and blood cultures was statistically significant.

Prevalence of infection, as determined by a combination of the kidney, urine, and blood cultures, was 17% for the opossum, 1.7% for the gray fox, 1.5% for the raccoon, and 1.0% for the striped skunk. A total of 107 or 4.4% of all animals tested was infected.

Trypanosomes from 5 opossums, 1 raccoon, and 1 striped skunk were studied and compared with *T. cruzi* and *T. rangeli* (Brooke et al, 1957). Isolates from the striped skunk and opossum were found to have *T. cruzi*-like characteristics. The isolate from the raccoon was more like *T. cruzi* than *T. rangeli*, but in culture appeared different from the other organism. Organisms of all other isolates, including those from the gray fox, were morphologically similar to known strains.

From the 89 animals which were found positive by direct culture inoculated with kidney tissue, trypanosomes were detected in the 4 types of media as follows: Chang's with rabbit serum, 44; Chang's with horse plasma, 27; Fletcher's with rabbit serum, 43; and Fletcher's with horse plasma, 63. Percentage recovery for the 2 base media was 40% for Chang's and 60% for Fletcher's; the difference was not statistically significant.

Trypanosomes were isolated from animals from 15 of the 22 study areas. Thirty percent of the infected individuals and 10% of the animals tested were collected on a single area in Calhoun County, Georgia. The reason for the high concentration of infection in the area is unknown.

DISCUSSION

The isolation of trypanosomes from kidney tissue and urine was secondary to the study of leptospirosis. Since most of the animals were tested by primary culture inoculated only with kidney tissue, and since 9 of the 107 isolates were obtained by other testing procedures involving approximately 350 (less than 20%) of the same animals, it is apparent that many infections were not detected. Infection rates for the various species are, therefore, only relative.

The known intermediate hosts of *Trypanosoma cruzi* in the United States are various species of triatomine bugs. In southwestern Georgia, *Triatoma sanguisuga* and *T. lecticularius* are the indigenous species but neither is abundant. Thirty-six wood rat nests were examined for these bugs, and only 3 were found. None was collected in traps operated for a week near a house where bugs had been found.

Contamination of mucous membranes or damaged skin with excreta or tissue from infected triatomine bugs is considered the usual method of transmission for *T. cruzi*. However, the high incidence of trypanosome infection in opossums is difficult to explain by this method in view of the relative scarcity of triatomine bugs.

Isolation of *T. cruzi* from the urine of opossums suggests the possibility of other methods of transmission. For example, transmission from mammal to mammal may occur through contact with urine containing viable organisms, especially during the mating period. Also, mammals may become infected by eating other infected mammals (Chandler, 1949).

Diamond and Rubin (1956) produced infections in 1-day-old pigs, lambs, and kids by inoculation of *T. cruzi*. Control animals housed with the infected ones remained free of trypanosomes during an experimental period of 4–8 weeks. Woody and Woody (1955) examined the urine of a child which had American trypanosomiasis 36 hours after onset of fever, and found it to be negative for trypanosomes. However, the shedding of viable trypanosomes in urine may be peculiar to the opossum and may explain the relatively high incidence of infection in this species.

No infected individuals were found in 8 of the species tested, but with the exception of the cottontail rabbit, the number of individuals tested was too small to provide valid information.

SUMMARY

A *Trypanosoma cruzi*-like organism was isolated from 93 of 552 (17%) opossums examined, 2 of 118 (1.7%) gray foxes, 9 of 608 (1.5%) raccoons, and 3 of 306 (1.0%) striped skunks from southwestern Georgia and northwestern Florida. Inoculation of kidney tissue in Chang's and Fletcher's base media with horse plasma or rabbit serum added revealed 103 infections of 1,974 animals tested. Of these, 98 were isolated in primary cultures and 5 from approximately 200 subcultures. The difference in the number of isolates obtained in primary cultures from the 2 base media was not significant.

Two infections were found by inoculating cultures with the urine of 27 opossums in Chang's and Fletcher's base media with horse plasma added. Nine isolates were obtained from trypanosome media inoculated with heart blood of 149 opossums; 13 isolates were obtained from 119 kidney tissue inoculations of the same animals. Six animals were positive by both techniques. Cultures inoculated with kidney tissue yielded a significantly higher percentage of infection than did cultures inoculated with blood.

Prevalence of infection, as determined by a combination of the isolates from kidney, urine, and blood, was 17% for the opossum, 1.7% for the gray fox, 1.5% for the raccoon, and 1.0% for the striped skunk. The prevalence of infection is relative only, since many infections probably were not detected by the methods used.

Isolation of trypanosomes from urine suggests that infections may be passed from mammal to mammal through contact with urine containing viable trypanosomes.

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RESEARCH NOTE

CHRONIC *TOXOPLASMA* INFECTION IN A HUMAN UTERUS

In connection with studies on the possibility of the congenital transmission of toxoplasmosis from chronically infected mother rats to their offspring, it was found that the uterus of such rats sometimes harbors the parasites, probably within pseudocysts (Remington, Jacobs, and Kaufman, 1958, J. Ophthalmol., to be published). A study of residual infection in the muscle of rats with chronic toxoplasmosis had been reported briefly earlier [Jacobs and Melton, 1957, J. Parasitol. **43** (Suppl): 41–42].

An extension of this work to human beings, to determine the presence of residual *Toxoplasma* infection in the uterus and other tissues of women dying of other causes, has been initiated. Portions of diaphragm, uterus, psoas muscle, and sometimes brain, are digested in pepsin according to the method of Jacobs and Melton [1957, J. Parasitol. **43** (Suppl): 38–39] and inoculated into mice. Evidence from other studies presently in progress indicates that the proliferative form of *Toxoplasma* is incapable of surviving digestion. Hence, any infections thus found may be presumed to be chronic.

Thus far 9 autopsy specimens have been obtained for this purpose. *Toxoplasma gondii* has been isolated from the uterus, as well as from diaphragm and psoas muscles, of a 57-year-old woman. This woman had a dye test titer of 1:256 but no evidence in her clinical history suggestive of toxoplasmosis; her death was sudden, due to coronary thrombosis. Histopathologic examination of tissues revealed no inflammatory process attributable to *Toxoplasma;* no parasites were seen. Although no cysts were seen, the clinical history as well as the fact that the parasites survived digestion indicates that we probably were dealing with a chronic infection.

This is the first report of the isolation of *Toxoplasma* from the uterus of a chronically infected woman. It has bearing on the hypothesis expressed in our earlier report (loc. cit.) that it is within the realm of possibility that infection of a foetus could result from the passage of parasites from a cyst in the uterine tissue, due to erosion of the tissue by the developing placenta. Some local proliferation of organisms released from a cyst could occur in the placenta even in the presence of antibody. If infections occurred in this manner, with passive transfer of antibody as well, the appearance of disease might be delayed in the offspring until some time after birth and might then be more limited in its manifestations. (Acknowledgment is gratefully made to Dr. Edward C. McGarry for providing the post-mortem material, history of the case, and pathological data.)—Jack S. Remington, Leon Jacobs, Marjorie L. Melton, *Laboratory of Tropical Diseases, National Institute of Allergy and Infectious Diseases,* and Herbert E. Kaufman, *Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, Bethesda, Maryland.*

VITAL STAINING OF BLOOD PARASITES WITH ACRIDINE ORANGE

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During the course of an immunological and immunochemical study of the dog heartworm *Dirofilaria immitis*, it was noted that the viable microfilariae were more easily seen under dark-field illumination. It was further noted that vital staining with acridine orange considerably enhanced this effect. The microfilariae rapidly absorbed the dye, staining a bright greenish yellow. Depending upon the concentration of the dye present, observed microfilariae became motionless in from 10 seconds to about 2 to 3 minutes. The possibility of examining stained, viable blood parasites prompted a study of the effects of acridine orange on the constituents of normal and parasitized whole blood.

MATERIALS AND METHODS

Aqueous dilutions of acridine orange (C.I. 788) up to 1:500,000 were prepared from a stock 1:5000 solution which was stored in the dark at room temperature. Blood was obtained from normal dogs, from dogs and hamsters which had been experimentally infected with pathogenic leptospires, and from dogs known to be infected with microfilariae of *D. immitis* or *Dipetalonema* spp. Blood samples also were obtained from canaries and white rats which had been experimentally infected with malarial parasites. Experimentally infected guinea pigs were the source for blood samples containing *Trypanosoma equiperdum* or *Toxoplasma gondii*. Leptospires and *Trypanosoma cruzi* from stock cultures were also examined. All blood samples were treated with heparin to prevent clotting.

Initial examinations were made with a Bausch and Lomb monocular microscope with a paraboloid dark-field condenser. A 100 w tungsten filament lamp was the light source; a Wratten blue violet (C-5) filter was used at the source when desired. Material also was examined with a Leitz Ortholux fluorescence microscope, equipped wiht a Leica 35-mm camera. A Phillips high pressure mercury lamp (CS-150) was the source of ultraviolet light. A primary B2 (blue-violet) or a Corning #5850 filter was used at the source and a yellow filter was used in the eyepiece.

EXPERIMENTAL

To 0.9 ml of canine blood was added 0.1 ml of an aqueous solution of acridine orange at a concentration of 1:5000. After mixing, a small drop was then placed on a clean glass slide, covered with a 22 mm × 40 mm coverslip in order to obtain a thin fluid layer, and sealed with Permount. The slide was then examined under dark-field illumination. Normal canary blood was similarly examined. At 150× magnification normal canine erythrocytes were not stained and presented a mesh-like appearance. However, the nucleus of the canary erythrocyte was conspicuously stained olive-green against the background of unstained cytoplasm. Leucocytes and platelets were distinctly visible as stained structures. The effects of this vital stain on the normal cellular elements of whole blood will be reported in detail elsewhere and therefore will not be dealt with here. Briefly, however, there was a rapid in vitro auto-differentiation of the leucocytes, permitting qualitative recognition, using 400 to 500× magnification.

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Since the cytoplasm of normal erythrocytes did not visibly absorb the stain, the appearance of parasitized erythrocytes in stained preparations was investigated. Blood from canaries experimentally infected with *Plasmodium relictum* was examined after dye had been added at concentrations of 1:10,000 and 1:50,000. A parasitized cell was easily distinguished because of the presence of a bright, white, refractile circular structure in the black cytoplasm of the cell. The olive-green oval nucleus was often eccentrically situated. Some cells contained 2, 3 or 4 structures in the cytoplasm. In most instances, the parasite was present in an erythrocyte which was larger than the normal cell. At 970× magnification, the white refractile structure appeared to be a part of a larger structure which was stained a very faint olive-green. At times, there was no visible cell outline and the parasite was extracellular (Fig. 1). Satisfactory differentiation of the leucocytes was also obtained with acridine orange at these concentrations.

FIGURE 1. Canary erythrocytes containing *Plasmodium relictum*. Components of the parasites appear as bright white refractile structures in the dark cytoplasm of the red cells. The insert at upper left demonstrates markedly the displacement of the nucleus.

Samples of blood were then obtained from white rats which had been experimentally infected with *P. berghei*. The slides were prepared as before and examined after addition of acridine orange to a final concentration of 1:10,000. With dark-field illumination it was easy to detect the parasitized cells within 2 minutes after the dye had been added to the blood. The parasitized erythrocytes were always larger than the normal cells and contained from 1 to 4 round refractile structures. The cell walls were often irregular and at times broken. Some cells contained masses of white refractile granules at one end, while in others a band of granules across the cell was observed. As many as 10 to 14 circular inclusion bodies were often evident in cells. Occasionally, an immature erythrocyte was identified by the round, bright green and eccentrically located nucleus. Also seen were numerous

extra-cellular groups of refractile structures and a typical demonstration of the process of exflagellation. Motility of *P. berghei* trophozoites persisted for as long as 4 to 5 days after the slide had been prepared (Fig. 2).

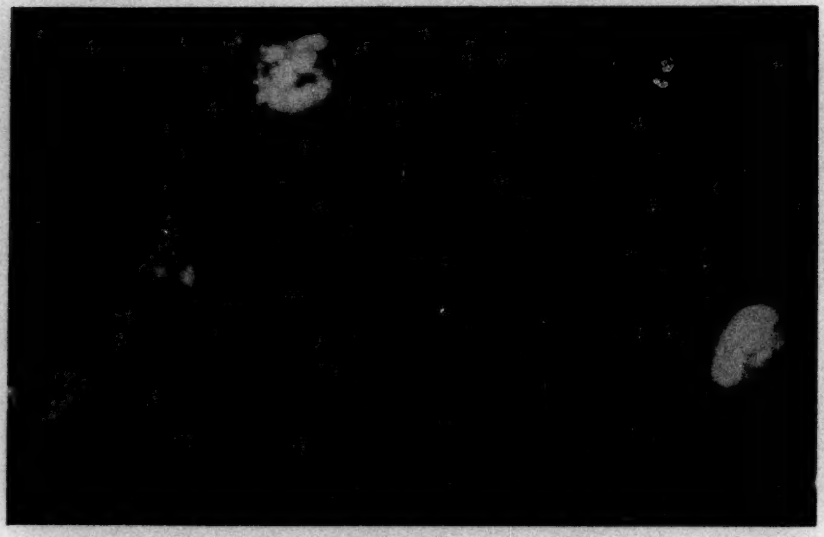

FIGURE 2. White rat erythrocytes containing *Plasmodium berghei*.

With the use of a Leitz Ortholux fluorescence microscope, examination of the materials described showed that the refractile structures seen before appeared to be integral components of a bright deep-red stained parasite. The nucleus of the cell, as in canary blood, maintained its green color, with a slight shift towards the yellow part of the spectrum. Variable morphology of the red-stained parasite from the circular inclusion forms described above to the irregularly shaped trophozoite forms was now apparent. Only 2 schizont-like stages were seen. The extracellular white refractile dots now appeared as red-stained structures.

It was noticed that the red color of the parasite became bleached by the mercury vapor lamp within 30 seconds, perhaps as a result of photo-oxidation. This reaction could be impeded by adding a Corning #5850 (blue-violet) filter to the system; shades of the original red color then persisted for as long as 4 to 6 minutes, thus making possible color photomicrography. It was then noticed that when a tungsten light source was substituted for the mercury vapor lamp bleaching did not take place. However, the illumination obtained with this high-intensity, low-voltage lamp was not too favorable for photographic reproduction.

Blood then was obtained from dogs which had been shown to be positive for microfilariae. The dye was added to a final concentration of 1 : 10,000 and slides were prepared as above. The microfilariae were stained within minutes and at 100× magnification, using the Bausch and Lomb microscope and the tungsten light source, rapid examination of thin suspensions revealed motile forms which became moribund within 10 seconds if kept in the microscopic field. This interval

of time was shown to be inversely proportional to the concentration of the dye. At the 1 : 500,000 concentration, motility ceased in approximately 2 minutes; the microfilariae appeared greenish yellow but were almost indistinguishable when the blue-violet filter was interposed between the light source and the microscope. Good differentiation was shown to take place when the dye concentration ranged from 1 : 3000 to 1 : 50,000, with motility ceasing from 5 seconds to 45 seconds respectively. There was no apparent deleterious effect on the microfilariae after the addition of the dye in all concentrations used, as long as the mixtures were not exposed to light. The microfilariae were viable for as long as 15 days after addition of the dye. Many underwent morphological change resembling the early changes which take place in the mosquito (Feng, 1936). They were viable for at least 3 to 4 days on a sealed slide in areas not exposed to the light source.

Thick suspensions were made by adding 25 to 45 mm^3 of the treated blood (1 : 10,000 to 1 : 50,000 dye concentration) to a slide and covering it with a 22×40 mm coverslip. The slide was scanned at $100\times$ magnification, using the filter system mentioned above with the tungsten lamp. These conditions eliminated the blanketing effect of the many erythrocyte layers which often completely covered platelets, white cells and microfilariae. At a magnification of $430\times$, a microfilaria extended approximately the entire diameter of the field (Fig. 3). Internal morphological differentiation was noted as the nuclei of the somatic cells were stained predominantly yellow, with some areas showing a faint pink to red while other areas were colorless. A well defined reddish-stained central body was distinctly seen when examination was made with the fluorescence microscope.

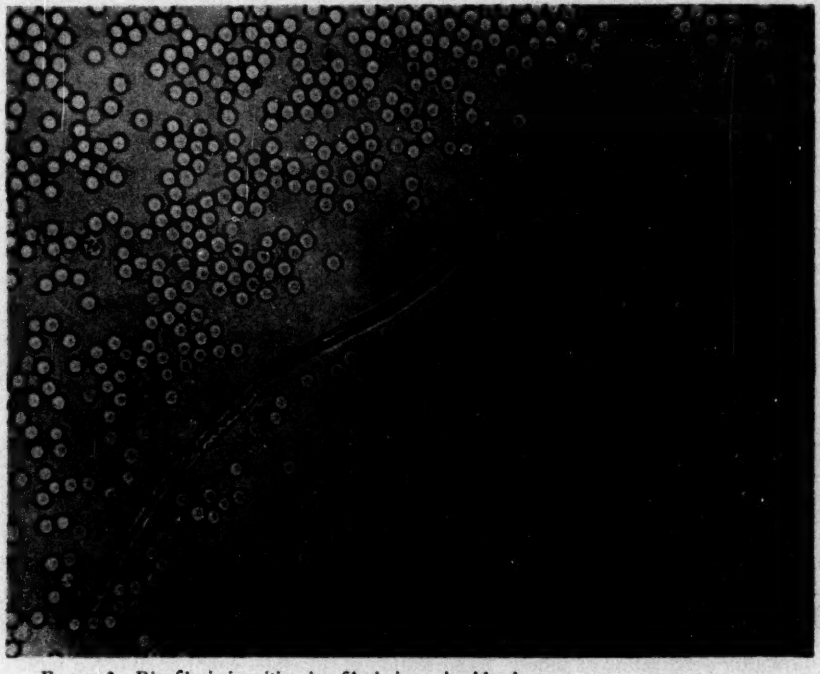

FIGURE 3. *Dirofilaria immitis* microfilaria in canine blood.

Guinea pig peritoneal fluid containing *Toxoplasma gondii* organisms was treated in the usual manner and the slides were examined. The minute half-moon-shaped parasite, at 970 × magnification, was yellow in color, and contained granules. With the blue-violet and yellow filters in the system it was most difficult to observe. However, very faint yellow and red round internal structures were noted. Viability was characterized by a side-to-side motion. Exposure for 5 minutes to microscopic illumination with the tungsten light source did not immobilize the organisms.

This also was true for culture suspensions of *Trypanosoma cruzi* containing a 1 : 10,000 concentration of dye, while as low as a 1 : 50,000 concentration of the dye was sufficient to immobilize *T. equiperdum* when blood of experimentally infected guinea pigs was treated with the dye alone.

The dye was added to whole culture suspensions of *Leptospira icterohemorrhagiae* in concentrations ranging from 1 : 250,000 to 1 : 12,500,000. The organisms were not inactivated when the tubes containing the various suspensions were kept in the dark. At the highest concentration of the dye, all motility ceased after a 2-hour exposure to daylight. After 18 hours of exposure to light all motility had ceased in the tube containing 1 : 1,450,000 concentration of the dye. A suspension of *L. sentot* containing a final concentration of 1 : 50,000 of the dye was examined at 900 × magnification with the fluorescence microscope. Motility ceased after 5 minutes of observation. The spirals were clear and sharp. Spaced approximately 2 microns from each end of the organism was an orange-stained round structure which was larger than the diameter of the organism, causing it to bulge.

DISCUSSION

Acridine orange was introduced by Bukatsch and Haitinger (1940) and Strugger (1940) for the vital staining of protoplasm. Strugger (1948) used it to make trypanosomes visible in the fluorescence microscope. DeBruyn et al (1950) showed that the dye could stain the nuclei of practically all the tissues of the living animal following parenteral or oral administration. It was then shown that the diaminoacridines have an affinity in vivo for intranuclear deoxyribose and ribosenucleoproteins. In vitro, they have an affinity for all nucleoproteins, regardless of their localization in the cell (DeBruyn et al, 1953). The dye has been used in radiobiology for the detection of radiation damage (Strugger et al, 1953). Venezian (1955) used it to study the metabolism of tobacco leaves. Others have used the fluorochrome to study its effect on spermatozoa (Duijn, 1954), on Ehrlich ascites and carcinoma cells (Weissman and Gilgen, 1956), on tubercle bacilli (Adamczyk, 1956) and also for the purification of viruses (Borecky, 1954).

Some disadvantages of the vital staining technique for blood smear examinations, as noted by others (Hall, 1938, and Wintrobe, 1956), are as follows: Smears could not be kept too long; cell nuclei and cytoplasm did not stain; erythrocytes could hide small lymphocytes; and finally, parasitic inclusions, especially the malarial organism, did not stain, except after long exposure periods to the dyes in use, and could easily be overlooked.

In reviewing the effects of acridine orange on the cellular elements of normal blood, it is pointed out that all cells, except erythrocytes, absorbed the dye. The cytoplasm and the nuclei stained rather distinctly, and the granules, when present, were quite noticeable. Viable parasites have been observed microscopically 4 to 6 days after preparation of slides. Examination of the slides could be initiated imme-

diately after the slides had been prepared. Preparations with thin-smear effects could readily be obtained merely by covering a small drop with a large coverslip, thus obviating the need of any particular skill for the streaking of thin smears. Stained blood cells and blood parasites could then be studied in the living state, permitting one to observe activity in the cell and to note the effects of external stimuli such as drugs or dyes, on the cells or on the parasites. The complex interrelations between the parasites and the affected cells, and the resulting pathological condition following parasitism are subjects of study to which direct microscopic observation can now be applied for longer periods of time. Actual observation of cell penetration by the stained parasite is possible, as well as the observation of phagocytic reactions of the leucocytes. Viable pseudopodia and long needle-thin stained protoplasmic nuclear extrusions have been observed.

Using a set of colored glass filters as an ancillary aid in the dark-field examination of whole blood, thick suspensions no longer presented a problem in the scanning for blood parasites. The multi-layer thickness of erythrocytes was completely neutralized, permitting one to examine rapidly field after field in the search for the stained parasites. The need for laking of the erythrocytes or for concentration of parasites by centrifugation has been eliminated. The observation that there is a specificity of reaction with respect to dye tolerance between 2 species of trypanosomes is of interest and merits further investigation.

The significance of the differentiation of the morphology of the leptospires as shown by this technique, is at present a matter of speculation. However, it should be noted that similar granules have been demonstrated by Molbert (1955), using the electron microscope. No detailed study has as yet been made to see if there is an internal cellular differentiation between the microfilariae of the genera *Dirofilaria* and *Dipetalonema* as a result of staining with acridine orange, or if there is a difference in the tolerance levels between the types of microfilariae. Since the lethal effect of the dye on *T. equiperdum*, as described above, was unique, this observation should be investigated more fully. The photodynamic inactivation of suspensions of leptospires also warrants further study to determine the suitability of vaccines or antigens prepared by this method.

The rapid in vitro auto-differentiation of the leucocytes of whole blood points to the possibility of studying the various blood dyscrasias using acridine orange and appropriate glass filters. This technique may facilitate a more detailed morphological differentiation of the leucocytes.

SUMMARY

Vital staining of the cellular elements of normal and parasitized whole blood with the basic fluorochrome acridine orange was shown to be a rapid and simple technique for examining, under dark-field illumination, differentiated leucocytes, and stained parasitized erythrocytes, containing the malarial organism in the presence of unstained normal erythrocytes. Microfilariae, trypanosomes, and *Toxoplasma* have also been stained in whole blood smears. Auxiliary use of glass filters has shown that the disadvantages of thick blood suspensions may be eliminated, permitting rapid observation of leucocytes and blood parasites. Photochemical inactivation of leptospires and microfilariae has been demonstrated. Differences in sensitivity or tolerance to acridine orange have been found in *T. cruzi* and *T. equiperdum*.

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PLATE I

1

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EXPLANATION OF PLATE

Photomicrography of Acridine Orange Stained Preparations.

Figure 1. Normal canine blood cells.

Figures 2, 3, 4. Microfilaria of the dog heartworm organism after 6, 4, and 9 days respectively in dog blood at room temperature.

Figure 5. Canary blood containing the malarial parasite.

EXPERIMENTAL INFECTION OF YOUNG PIGS FOLLOWING INTRANASAL INOCULATION WITH NASAL, GASTRIC, OR CECAL TRICHOMONADS FROM SWINE OR WITH *TRICHOMONAS FOETUS*

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Although it is recognized that parasites of the genus *Trichomonas* are of common occurrence in the nasal cavity, stomach, and cecum of swine, only 1 species has thus far been named. *Trichomonas suis* was the name given to trichomonads from the stomach of pigs by Gruby and Delafond (1843) and Davaine (1877). Switzer (1951) reported that trichomonads were often found in the nasal cavity of swine in association with atrophic rhinitis. Buttrey (1956) observed that trichomonads found in the nose of swine are similar, morphologically, to *T. foetus* and that 2 types of trichomonads occur in the cecum of swine, both of which are morphologically distinct from the nasal trichomonad.

The taxonomic relationships of the various trichomonads that parasitize swine have not been determined. The investigation herein reported was designed to procure information that would assist in elucidating the taxonomic relationships of the various trichonomads that occur in the nasal tract, stomach, and cecum of swine, and their relation to *T. foetus*.

METHODS

Pigs used in the experiments herein reported were separated from the sow immediately following birth. Natural birth was allowed in the case of pigs involved in experiments 1 and 2, whereas the pigs were taken by caesarean section in experiment 3. Pigs in experiment 1 were housed in stainless steel wire pens, while those in experiments 2 and 3 were kept on concrete floors covered with wood shavings.

All pigs were bottle-fed at intervals of 3 hours until old enough to drink from a pail. Newborn pigs in experiment 1 were fed a commercial feed preparation mixed with water and homogenized cow's milk plus concentrated ferric sulfate solution. The milk was discontinued after 3 weeks. In experiments 2 and 3, feed for the newborn pigs consisted of a mixture of 1 pint of homogenized cow's milk, 1 pint of lime water (0.2%), ¼ pint of cream, and ¼ pint of Karo syrup. Ferric sulfate and a preparation containing vitamins A, B, C, D, and E were added to the mixture at each feeding. Between ages 10 days and 3 weeks, the commercial feed preparation was gradually substituted for the above mixture. Eight of 17 pigs survived until the time of inoculation in the first experiment, 8 of 8 in the second, and 13 of 22 in the third.

When the pigs were 6 to 8 weeks old, they were segregated into 4 or 5 groups and inoculated intranasally with trichomonads. The groups were separated by solid partitions so that contact between pigs of different groups was prevented. Three to 5 weeks after inoculation the pigs were killed and examined for trichomonads.

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EXPERIMENTS

Experiment 1. In this experiment, involving 8 pigs, the first inoculation was made when the pigs were 42 days old; and the inoculation was repeated 3 times during the following week. Two pigs, the controls, received an inoculum of physiological saline only, another was inoculated with porcine nasal trichonomads, 2 with porcine gastric trichomonads, 2 with porcine cecal trichomonads, and 1 with *T. foetus*. The trichomonads had been grown in modified Plastridge beef extract medium as described elsewhere (Fitzgerald et al, 1954). The cultures had been maintained in the laboratory for 9 to 12 months prior to their use in this experiment. Five to 10 ml of culture fluid containing an estimated 1 to 3 million trichomonads per ml were given in each inoculation. After an interval of 12 to 24 days from the last inoculation, the pigs were necropsied and the nasal cavity, stomach, and cecum examined for trichomonads by direct microscopic examination and by culture. The same culture medium was used for this purpose as for the inoculum. The results are shown in Table I.

TABLE I.—*Results of intranasal inoculations of young pigs with cultures of trichimonads from the nose, stomach, and cecum of swine, or with cultures of* T. foetus.

Group	Pig No.	Age in Days at Inoculations	Age in Days at Examination	Results of Examination for Trichomonads		
				Nose	Stomach	Cecum
Control	5*		62	–	–	–
Porcine nasal	6	42, 43, 46, 49	73	–	–	+**
Porcine gastric	7	42, 43, 46, 49	70	+**	–	+ *Retortamonas* sp. found in cecum
	8	42, 43, 46, 49	73	+	+**	+**
Porcine cecal	2	42, 43, 46, 49	61	–	–	+
	3	42, 43, 46, 49	63	–	–	+ *Retortamonas* sp. found in cecum
T. foetus	4	42, 43, 46, 49	73	–	–	+**

* A second control pig died before completion of the experiment.
** Positive by culture but not by direct microscopic examination.

Experiment 2. Pigs in this experiment were inoculated with fresh material containing trichomonads collected from the nasal and gastro-intestinal tracts of swine at the time of slaughter in local abattoirs. This procedure was adopted in order to eliminate any possible influence of culturing on the organisms. Eight pigs were used, 2 of which (the controls) were uninoculated. Each of the test pigs received 2 or 3 inoculations during a period of approximately 1 to 3 weeks. The pigs were 9 days old at the time of the first administration of material containing trichomonads.

Because of the difficulties encountered in obtaining suitable inocula, the various groups of test pigs were inoculated at different times. The longest delay occurred in the case of pigs inoculated with material from the stomach; in this case, the last inoculation was administered when the pigs were 56 days old. The materials used for inoculation included nasal washings collected just before slaughter, also stomach and cecal contents obtained just after slaughter. After microscopic examination of the samples, from 5 to 10 ml of each type of material was inoculated intranasally into the test pigs within 4 to 5 hours of collection. The pigs were killed and examined for trichomonads, as in the first experiment, 15 to 29 days after the last inoculation. The findings are summarized in Table II.

TABLE II.—*Results of intranasal inoculations of young pigs with trichomonads contained in fresh material from the nose, stomach, and cecum of pigs.*

Group	Pig No.	Age in Days at Inoculations	Age in Days at Examination	Results of Examination for Trichomonads		
				Nose	Stomach	Cecum
Control	3		63	–	–	–
	7		63	–	–	–
Nasal washings	2	16, 23, 35	63	+	–	+
	8	16, 23, 35	63	+	–	+
Stomach contents	5	37, 45, 56	71	+	–	–
	6	37, 45, 56	71	+	–	–
Cecal contents	1	9, 16	45	+	–	+
	4	9, 16	45	–	–	+

Experiment 3. Thirteen pigs were used, 3 of which served as uninoculated controls. The others were first inoculated with trichomonads from culture when the animals were 52 to 64 days of age. An additional inoculation was administered about 4 days later. Three of the pigs were inoculated with porcine gastric trichomonads, 2 with porcine cecal trichomonads, and 2 with trichomonads from the nasal cavity; 3 were inoculated with *T. foetus*. At each inoculation, 10 ml of culture fluid containing an estimated 1,500,000 to 4,000,000 trichomonads per ml was administered.

The trichomonads from the stomach and cecum of pigs had been cultured in CPLM medium; those of nasal origin and the culture of *T. foetus* had been maintained in modified Plastridge medium. All cultures except those of the stomach trichomonad had been freed of bacteria by the use of U-tubes and antibiotics.

The organisms used had been maintained in culture for a relatively short time in order to minimize the possibility of changes in virulence. For example, the cultures of porcine trichomonads had been isolated 4 or 5 days prior to the initial inoculations and had undergone only 3 or 4 transfers. The strain of *T. foetus* had been isolated from an infected bull about 6 weeks prior to use, and the culture had undergone 15 transfers prior to its use.

After the inoculations, fecal samples were collected from the pigs at regular intervals and examined for trichomonads. The animals were killed 18 to 20 days after the last inoculation. Samples from the nasal passages, stomach, middle portion of the small intestine, and from the cecum and colon were examined for trichomonads. Modified Plastridge and CPLM media were used to supplement the direct examinations.

Trichomonads were found in the feces for 1 to 2 days after inoculation with nasal trichomonads, for 4 days after inoculation with stomach trichomonads, and for 6 days after inoculation with cecal trichomonads and with *T. foetus*. The findings at necropsy are shown in Table III.

No consistent differences in morphology as revealed by study of living specimens could be observed in the trichomonads from the different sites in the variously inoculated pigs.

TABLE III.—*Results of intranasal inoculations of young pigs with cultures of trichomonads from the nose, stomach, and cecum of swine, or with cultures of* T. foetus.

Group	Pig No.	Age in Days at Inoculations	Age in Days at Examination	Results of Examination for Trichomonads				
				Nose	Stomach	Small Intestine	Cecum	Colon
Control	1		74	–	–	–	–	–
	2		74	–	–	–	–	–
	3		88	–*	–	–	–	–
Porcine nasal	4	52, 56	74	+++	–	–	++	–
	5	52, 56	74	++++	–	–	++	–
Porcine gastric	6	64, 68	88	+++*	+	+	+++	++
	7	64, 68	88	++*	–	+	++	+
	8	64, 68	88	++*	–	–	++	+
Porcine cecal	9	52, 56	74	+++	+	–	++	–
	10	52, 56	74	++	–	–	++	–
T. foetus	11	52, 57	74	–*	–	–	+	–
	12	52, 57	74	–	–	–	+	–
	13	52, 57	88	–*	–	–	+	+

\+ = Fewer than 3 trichomonads per low-power field;
++ = 3 or 4 trichomonads per low-power field;
+++ = 5 to 10 trichomonads per low-power field;
++++ = More than 10 trichomonads per low-power field.
* Organisms other than trichomonads found, including a species of nematode and several species of protozoa.

DISCUSSION

Evidence against the possibility that extraneous infections with trichomonads occurred in the pigs used in the investigation herein reported is supplied by the fact that the control (uninoculated) pigs were free of these parasites at necropsy insofar as could be determined. Moreover, because of the lack of contact between these pigs and other pigs before and during the experiment, it is assumed that the infections found in these animals resulted only from the inoculations.

Assuming this to be so, a question remains as to the nature of the inocula used. It is possible that the inoculum of trichomonads from the cecum may also have contained trichomonads from the stomach and/or nose, that the inoculum of stomach trichomonads included trichomonads from the nose, and that the inoculum from the nose also contained cecal trichomonads.

The findings might, therefore, be explained on the basis that each inoculum contained trichomonads of more than one type. Alternative explanations are that the trichomonads from the different sites are able to become established at sites other than the one in which they are usually found, or that the trichomonads from the various sites are varieties of the same species. Morphological and physiological data must be correlated with those from transmission studies such as those herein reported in order to solve these problems.

It is of interest that trichomonads were found in the cecum of all inoculated pigs except 2 inoculated with stomach contents. The failure of trichomonads to become established in the cecum in these 2 cases may be associated with the relatively small number of trichomonads in the inoculum. In 2 of the 3 experiments trichomonads were found less frequently in the nose than in the cecum, although the nose was presumably more thoroughly exposed to infection than any of the other sites as a result of the intranasal method of inoculation.

The finding of trichomonads in the stomach of only 3 pigs in the 3 experiments indicates that it is more difficult for trichomonads to become established in the stom-

ach than in the cecum and nasal passages. This agrees with the relatively low incidence of natural infections with trichomonads in the stomach (Hammond, Fitzgerald, Johnson, 1957).

The finding of *T. foetus* in fewer sites and in smaller numbers than the trichomonads of porcine origin observed in experiment 3 may possibly have been influenced by the fact that the trichomonads of bovine origin had been maintained in culture considerably longer than the porcine forms. In agreement with this interpretation is the evidently greater virulence of the more recently isolated porcine trichomonad cultures in the third experiment as compared with those of the first.

The apparent ability of *T. foetus* to become established in the cecum of the pig is worthy of note. In this connection it has been reported that, under experimental conditions, *T. foetus* is able to become established in the vagina of sheep (Andrews and Rees, 1936; Fitzgerald, Johnson, Hammond, unpublished) and of goats and swine (Hammond and Leidl, 1957). It has also been shown that trichomonads from the nose or digestive tract of the pig may become established in the genital tract of cattle (Switzer, 1951; Fitzgerald et al, 1955; Hammond and Leidl, 1957) and of sheep (Fitzgerald, Johnson, Hammond, unpublished). On the basis of the cited work and that of the authors it would appear that these trichomonads have a relatively low degree of organ and/or host specificity.

A conclusion as to the identity of the trichomonads of the nose, stomach, and cecum of pigs must await completion of morphological studies now in progress.

SUMMARY

In each of 3 experiments, 7 to 13 pigs 9 to 52 days old were divided into groups and were inoculated 2 to 4 times with sterile saline solution, or cultures or fresh material containing trichomonads from the nose, stomach, or cecum of pigs or with *Trichomonas foetus*. The pigs were killed 15 to 29 days after the last inoculation and examined for trichomonads.

No trichomonads were found in any of the uninoculated (control) pigs. Of 5 pigs inoculated with nasal trichomonads, trichomonads were found in the noses of 4 and in the ceca of all. Of 7 pigs inoculated with gastric trichomonads, trichomonads were found in the noses of 7, in the stomachs of 2, and in the ceca of 5. Of 6 pigs inoculated with cecal trichomonads, trichomonads were found in the noses of 3, in the stomach of 1, and in the ceca of 6. Of 4 pigs inoculated with *T. foetus*, trichomonads were found in the ceca of all. In addition, trichomonads were found in the small intestine of 2 of 3 pigs inoculated with gastric trichomonads, and in the colon of all 3 of these pigs and of 1 of 3 pigs inoculated with *T. foetus*.

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RESEARCH NOTE

FURTHER OBSERVATIONS ON THE EXPERIMENTAL HOST-PARASITE RELATIONS OF THE GUINEA PIG AND THE RUMINANT PARASITE, *TRICHOSTRONGYLUS COLUBRIFORMIS*

It has been shown recently that the guinea pig can be experimentally infected with *Trichostrongylus colubriformis*, a nematode parasite of ruminants (Herlich et al, 1956, Proc. Helminth. Soc. Wash. **23**: 104–105). Additional experiments have been conducted in an attempt to determine whether or not sex and age of the host may influence its susceptibility to infection, and to determine if the nematode is pathogenic to the guinea pig.

In each of 2 experiments, 10 male and 10 female guinea pigs that were 20½ weeks old and the same number that were 11½ weeks old were each administered per os approximately 4,150 infective larvae of *T. colubriformis* cultured from eggs in feces collected from a calf experimentally infected with this nematode. Thirteen days after administration of the larvae, diarrheic feces were passed by 1 of the older males, 1 of the older females, and half of the younger males. Feces of the remaining guinea pigs remained normal in consistency. One of the older females and 1 of the younger males died on the 14th day of infection, and all remaining animals were killed at that time. The numbers of worms recovered ranged from 20 to 4,085 per animal, with 40 guinea pigs harboring fewer than 500 worms (10% of the infective larvae administered) and 15 guinea pigs more than 2,075 worms (50% of the infective larvae administered). Adolescent and sexually mature worms were recovered. Extreme variation in worm number occurred in each age and sex grouping, and, in general, those animals harboring the most worms made the poorest weight gains or actually lost weight, while those harboring the fewest worms made the best gains. In experiment 1, the younger females gained an average of 7.6 g, whereas the other 3 groups averaged losses of from 1.8 to 29.2 g. In the second experiment, 3 of the groups gained an average of from 29 to 51 g, whereas only the older male group showed a loss in weight that averaged 17.4 g.

Since the foregoing results indicated that 4,150 infective larvae were capable of producing detrimental and even lethal effects in guinea pigs, another experiment was conducted in which 5 male and 5 female guinea pigs that were 8 weeks old were each given 10,000 infective larvae. One male died 18 days after infection, and 5,444 sexually mature worms were recovered from its small intestine, cecum, and colon. Two females dying 15 and 25 days after infection harbored 4,080 and 2,700 worms, respectively. Adolescent and sexually mature worms were found in the female dying at the earlier time, while only mature worms were recovered from the other female. The 7 surviving animals were killed 25 days after infection, and the numbers of worms still present ranged from 1 to 1,034, all sexually mature. One female had no worms at the time of necropsy. Of the 4 surviving males, 2 had lost 67 and 80 g, whereas the other 2 had gained 4 and 49 g. On the other hand, gains of 65, 76, and 76 g had been made by the 3 surviving females.

Within the limits of these few trials, the results indicate that (1) the susceptibility of the guinea pig to infection with *T. colubriformis* is highly variable, (2) sex and age of the host apparently had little or no effect on susceptibility to infection, and (3) *T. colubriformis* is detrimental to the health of guinea pigs with lethal infections resulting from the administration of from 4,150 to 10,000 infective larvae.—HARRY HERLICH, *Regional Animal Disease Research Laboratory, Agricultural Research Service, U. S. Dept. of Agriculture, Auburn, Alabama.*

THE MECHANISM OF TRANSMISSION OF *HAEMOBARTONELLA MURIS* (MAYER) OF RATS BY THE SPINED RAT LOUSE, *POLYPLAX SPINULOSA* (BURMEISTER)*

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Normal adult albino rats infected with *Haemobartonella muris* (Mayer) show no evidence of disease. However, splenectomy results in a severe, often fatal, anemia of the macrocytic hypochromic type.

Ford and Eliot (1928) first suggested transmission of the disease by an insect vector when they noted that all of their rats were infested with the spined rat louse, *Polyplax spinulosa* (Burmeister). A few months later, Cannon and McClelland (1928, 1929) showed that lice could transmit *H. muris* directly from rats with latent infections to uninfected rats. Mayer (1928b) achieved similar results independently and there was further confirmation by Eliot and Ford (1929) and Vassiliadis (1930).

The first attempt to determine the mechanism of transmission by lice was made by Eliot and Ford (1929). They fed 10 lice to a splenectomized Wistar rat which remained uninfected after 3 months of observation. It was not reported whether these lice came from an infected source. They also tested transmission by the bites of the vectors. They placed lice from infected animals on splenectomized bartonella-free rats which subsequently developed typical anemias. Similarly, Timmerman (1930) transferred lice from an infected rat to a splenectomized white rat but allowed them to feed through muslin. This rat later became positive. Modes of transmission other than by the bite were not excluded in these experiments. Consequently, a study was initiated to determine more precisely the mechanism of transmission.

MATERIALS AND METHODS

The animals used throughout the experimental work were derived from Wistar rats which are free of latent bartonellosis but highly susceptible to infection (Cannon and McClelland, 1928, 1929; Eliot and Ford, 1929; Crystal, 1956). Where possible, mature rats for each experiment were selected for similarity of sex, age and weight. Test animals were isolated individually for the duration of the experiment. Experimental rats were splenectomized within 3 weeks after exposure to test materials (Crystal, 1956). Erythrocyte counts, hemoglobin determinations and the examination of Giemsa-stained films for bartonellae were made from tail blood daily for at least 15 days.

H. muris was obtained from wild rats trapped in Berkeley, California, and was maintained by intraperitoneal inoculations of infected cardiac blood into healthy animals.

Two separate colonies of uninfected and of infected *P. spinulosa* were maintained. It was shown that this strain could readily convey *H. muris* from one rat to another (Crystal, 1956).

Each experiment was controlled by inoculations of rats with saline suspensions of lice and by injections of physiological saline. Unless otherwise stated, all inoculations were performed via the intraperitoneal route.

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RESULTS

Attempts to transmit H. muris *by the ingestion of infected lice by rats.*

Each of 3 rats was fed a saline suspension of 50 crushed lice from infected rats. The lice were contained in a bolus of bread or were fed to the rats by means of a tube. At the same time, 50 or 60 lice from the same host rats were inoculated into each of 3 other rats and 50 additional lice were placed on each of 3 separate rats. Another rat was not treated. All of the 3 control rats exposed to lice and the 3 injected parenterally showed infections after splenectomy. The rats which ingested lice and the untreated rat revealed no bartonellae or significant anemia.

Attempts to transmit H. muris *by the deposition of feces from infected lice on the skin of rats.*

Feces were collected from 3 lots of 50, 200, and 281 lice respectively, each lot from an infected rat. Each fecal collection was suspended in physiological saline and inoculated into a rat. The 3 groups of lice were inoculated individually into other rats. Three rats received inoculations of physiological saline. All animals were splenectomized 2 weeks later. Only the 3 rats inoculated with lice developed bartonellosis. Several thin smears, made of the feces collected for 2 hours from 200 infected lice, did not reveal the presence of any bartonella-like structures or Gram negative organisms.

Transmission of H. muris *by rubbing crushed lice into the scarified skin of rats.*

On 4 occasions, physiological saline suspensions were prepared of 25 or 50 triturated lice from 4 rats with latent infections. Each time, one suspension of 25 lice was inoculated into a rat. In addition, suspensions of 25 or 50 lice were rubbed into the scarified skin of 6 rats. Physiological saline alone was rubbed into the scarified skin of 4 other rats. Three of the 4 rats inoculated intraperitoneally with lice developed bartonellosis following splenectomy. Two of the rats inoculated with 25 lice via the scarified skin and 2 inoculated with 50 lice revealed bartonellae after splenectomy. The saline control animals did not show any sign of infection.

Transmission of H. muris *by the bite of the louse.*

Five hundred lice from 2 rats with latent infections were starved for 24 hours. After this period, 344 of the lice had died. These plus 52 of the survivors were proved infected by inoculation into a rat which became positive for *Haemobartonella* following subsequent splenectomy. Sixty-two additional surviving lice were placed on the skin of a rat which was prevented from biting or scratching its body for 2 weeks by a restraining jacket (Crystal, 1956). Following the removal of the restraining jacket, the rat was splenectomized. Immediately thereafter, cardiac blood was withdrawn and inoculated into a third rat in order to determine if the restrained rat was infected at this time. The restrained test animal died within 15 minutes after cardiac puncture probably due to the combined effects of prolonged restraint and surgical intervention. The rat inoculated with blood and a control rat injected with saline only remained healthy.

Three hundred lice from a carrier rat were placed in a feeding capsule (Jellison and Philip, 1933) closely applied to the skin of a rat. Each pair of legs of the rat was hobbled and a collar was fitted around the neck of the rat to keep it from disturbing the capsule. After 4 hours, the lice were recovered and starved for 20 hours. Of the surviving lice, 144 were placed in a feeding capsule on a second rat. Four hours later, all the lice were recovered from this rat. There were 43 alive and all but 4 of these lice had fed. The dead and living lice were pooled and inoculated into

another rat. A fourth rat received an inoculation of saline only. The first test animal, on which the lice were fed immediately upon removal from the carrier rat, developed bartonellosis. Similarly, the rat inoculated with lice succumbed to the infection. Neither of the remaining rats developed bartonellosis.

DISCUSSION AND CONCLUSIONS

Mayer (1928a), Eliot and Ford (1929) and Sorge (1929) fed liver, lice or other material from infected rats to presumably bartonella-free rats. Except for a single positive result obtained by Mayer, they were not able to establish infection by this method. However, Mayer did not give adequate proof that his test animal was not already infected with bartonellae. In view of the weight of both past and present evidence, it may be concluded that transmission of *H. muris* by the ingestion of infected lice does not take place.

Weinman (1944) suggested that *H. muris* may be excreted in the feces of infected lice. This hypothesis was tested in the current study. The experimental data show that *H. muris* is not voided in the fecal deposits of *P. spinulosa*.

The evidence indicates that *H. muris* may pass from the crushed bodies of infected lice through the broken skin. Under natural conditions, rats infested with lice show numerous superficial abrasions of the skin. Certainly, in attempting to free itself of its ectoparasites, the rat crushes some lice. Another possible portal of entry for *H. muris* from crushed lice is the buccal mucosa. Infested rats frequently try to bite the lice in order to remove them from the skin. However, Sorge (1929) was not able to establish infection by the instillation of the conjunctival sac with infectious material. The organism may be able to pass only through the broken mucous membrane.

It has been suggested by Cannon and McClelland (1929), Eliot and Ford (1929), Mayer (1928b) and Timmerman (1930) that the mechanism of transmission of this infection may be by the louse biting the healthy rat and inoculating bartonellae through its mouthparts. Eliot and Ford (1929) transmitted the infection by placing lice from infected animals on splenectomized bartonella-free rats. Since transmission may take place from crushed lice, the experiments of Eliot and Ford do not conclusively demonstrate the transmission of *H. muris* by the bite of the vector. On the other hand, Timmerman (1930) fed lice on an infected rat for 1 hour and then on the shaved belly of a splenectomized white rat through muslin for ½ hour. The rat subsequently developed bartonellosis. The brief interval of time between the infecting and infective feedings of the lice was hardly enough for any significant multiplication of *H. muris* to have occurred. Mechanical transmission by the bites of lice which have recently left an infected host cannot be excluded. However, since the lice were obtained originally from a wild rat, they may not have been uninfected when they were fed for the first time. If this were the case, it would not permit of a choice between mechanical transmission and biological transmission. The present experimental data suggest that *H. muris* is transmitted mechanically but not biologically by the bite of *P. spinulosa*. The location of the parasite in the louse is unknown; the writer has not been able to detect *H. muris* in serial sections of presumably infected lice. It may be necessary to seek an extra-alimentary location of *H. muris* in the louse.

It appears that transmission normally occurs by the rat crushing infected lice and inoculating itself by scratching and biting. Transmission may also occur directly; the louse feeding on an infected rat and then proceeding immediately to feed on a second rat may inoculate bartonellae remaining on its mouthparts.

SUMMARY

1. Susceptible albino rats did not become infected with *Haemobartonella muris* (Mayer) as a result of ingesting crushed *Polyplax spinulosa* (Burmeister) from an infected source when administered in a bolus of bread or by tube.

2. The intraperitoneal inoculation of susceptible albino rats with the feces of infected *P. spinulosa* did not result in infection with *H. muris*. Stained smears made from such feces did not reveal the presence of organisms resembling *H. muris*.

3. When *P. spinulosa* from an infected source were crushed and rubbed into the scarified skin, infection with *H. muris* followed.

4. *P. spinulosa* from an infected source, maintained for 24 hours without access to bartonella-containing blood, did not produce infection with *H. muris* by their bites when placed on susceptible albino rats. The bites of infected *P. spinulosa* allowed to feed upon a susceptible albino rat for 4 hours immediately following their removal from an infected rat did produce infection.

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THE ANTHELMINTIC EFFECT OF BEPHENIUM ON *ANCYLOSTOMA CANINUM*

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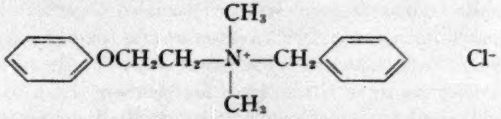
A new series of anthelmintics, designated by the generic name, bephenium, has shown activity against a large number of intestinal nematodes of laboratory and domestic animals. These compounds appear to be more active against nematodes attached to the mucosa than to those living free within the lumen (Copp et al, 1958). One of the compounds, bephenium embonate, was found effective against larval and adult forms of *Nematodirus* in lambs (Rawes and Scarnell, 1958).

Bephenium chloride has the following formula:



Bephenium bromide has Br^- instead of Cl^-; bephenium iodide has I^-; and bephenium hydroxynaphthoate has $C_{11}H_7O_3^-$. Bephenium embonate, which was quite effective against *Nematodirus*, showed a variable activity, probably due to its greater insolubility even when finely milled, against *Ancylostoma caninum*, and is not included in the following study.

PROCEDURE

All mongrel dogs and cats received by our Laboratory were examined for helminth infections and 50 of those showing hookworm eggs in the stools were selected for treatment. All of these animals (15 dogs and 35 cats) had natural infections of *Ancylostoma caninum*, with an average of 10 to 12 hookworms per animal; the heaviest infection found was of 60 hookworms.

All dosages were computed as mg of base per kg of weight. The compounds were administered in gelatin capsules and generally given about 8 A.M. The animals were fed at the usual feeding time, about 1 P.M. No pre- or post-treatment purges were given. All feces were collected for 48 hours after dosing, were washed through a 40-mesh sieve; the material remaining in the sieve was poured, a little at a time, into a black-bottom pan and examined for worms.

At the end of 48 hours after dosing the animals were killed and the entire intestine removed. The large and small intestines were separated, put in different dishes, and each slit lengthwise. The contents of the lumen were washed into a dish and the mucosa was examined twice carefully for any attached worms, which were removed and placed in the dish. The contents of the two portions of the intestine were washed separately through the sieve and examined in the black pan as was done with the stool specimens. The contents of the large instestine were considered as part of the second 24-hour stool specimen, insofar as worms other than *Trichuris* were concerned.

RESULTS

Ancylostoma caninum. The majority of the hookworms were killed by the various salts, regardless of the size of the dosage (Table I). Single doses of over 20 mg/kg resulted in the elimination of 99.4% of the hookworms and the clearance of 17 of the 19 animals. Each of the two not cleared had a single living hookworm at autopsy. Single doses of 20 mg/kg or less caused the loss of 94.1% of the hook-

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worms and cleared 22 of the 31 animals. Seven of the 9 not cleared were found to have only a single hookworm remaining at autopsy.

In general, stool specimens passed 3 hours or more after dosing contained hookworms, and in several animals all or the majority of hookworms were passed in less than 7 hours after dosing. For example, Cat 53 passed all 7 and Cat 47 all 8 within 3 hours; Cat 1, 10 of 17 in 3 hours; Cat 14, 40 of 52 in 5 hours; Dog 10, 15 of 35 in 5 hours; Cat 75, 44 of 57 in 5 hours; and Cat 4, 15 of 16 in 6 hours. Of all the hookworms killed by these compounds, 97.3% were eliminated during the first 24 hours after dosing.

Hookworms passed during the first 6 hours after dosing were generally contracted and/or twisted. Those passed later apparently had relaxed in death and were less distorted. The compounds appear to have killed the worms quickly, for it was not unusual to find dead worms still in copulation.

Toxocara cati and *T. canis*. These two species responded in a similar manner to the bephenium compounds and will be discussed together. The percentage of worms eliminated varied considerably, even at the same dosage level. However, there was a higher rate of loss with the larger dosages. In the 7 animals receiving bephenium in a single dose of 50 mg/kg or higher there was a 66.6% loss of worms. In the 17 animals receiving a single dose of less than 50 mg/kg, a 24.3% elimination was obtained.

Trichuris vulpis. Bephenium chloride was administered to 3 dogs at 50 mg/kg daily for 5 to 6 days, but was not partciularly effective, as there was only 10.5% loss of whipworms. Two other dogs received enemas of 50 and 100 mg/kg of the chloride and lost only 3.2% of their worms. Due to the fact that practically all of the whipworms are found in the distal portion of the cecum, or appendix, the compound apparently does not reach the worms or reaches them only in minute amounts.

Physaloptera sp. Various species of *Physaloptera* are found in carnivores, monkeys and man, where they are generally attached to the stomach. Dog 39 was found to have *Physaloptera* sp., and was given 50 mg/kg of bephenium chloride in 100 ml of water as a single dose by stomach tube. One worm was passed during the first 24 hours, 2 during the second 24 hours and 3 were found at autopsy, giving a clearance of 50%. As this worm appears to respond to bephenium, it is probable that 2 or 3 daily doses would be better than giving a single larger dose.

TABLE I.—*Action of single doses of bephenium compounds against* Ancylostoma caninum.

Salt	Dosage mg (base)/kg	No. Animals Treated	No. Animals Cleared	% of Worms Eliminated
chloride	25 – 100	10	10	100.0
	15 – 20	3	3	100.0
	10	3	2	93.7
	2.5 – 5.0	4	1	61.6
bromide	15 – 20	2	2	100.0
	10	4	4	100.0
iodide	45 – 67	6	5	99.5
	15 – 20	2	1	93.9
	10	2	1	85.7
hydroxynaphthoate	30 – 50	3	2	97.5
	15 – 20	4	2	94.1
	10	3	3	100.0
chloride + hydroxynaphthoate	5 + 10*	3	2	94.1
	10 + 10	1	1	100.0
TOTAL		50	39**	97.1

* The first figure refers to the chloride and the second to the hydroxynaphthoate.
** Of the 11 animals not cleared, nine had only a single hookworm remaining at autopsy.

Tapeworms. Bephenium compounds had no effect on the 15 *Dipylidium caninum*, 2 *Taenia pisiformis* and 1 *Hydatigera taeniaeformis* infections which occurred in this group of cats and dogs.

In vitro experiments. Various dilutions of bephenium chloride in Ringer's solution were prepared and kept at 37° C in the incubator. Worms put into these solutions were observed at frequent intervals and when they appeared dead, they were transferred to drug-free Ringer's solution to see if they would recover. *Ancylostoma caninum* died in 15 to 20 minutes in a 1 : 100 dilution and in 25 to 43 minutes in a 1 : 200 dilution. In a 1 : 500 dilution they still showed a faint trace of movement after 8 hours, but died after being transferred to drug-free Ringer's. *Toxocara cati* died in 60 to 75 minutes in a 1 : 100 dilution and in 5.5 to 6.5 hours in a 1 : 200 dilution. *Trichuris vulpis* was more resistant than either of the others, requiring from 2 to 7 hours in a 1 : 100 dilution. In none of these experiments did worms recover when transferred to drug-free Ringer's.

Effectiveness of bephenium. As hookworms generally are attached to the duodenum slightly posterior to the pyloric valve, they probably come in contact with the bephenium compounds in concentrations greater than 1 : 200 and are killed rather quickly. Those hookworms attached farther down apparently come in contact with a more dilute solution of the compound and those which survive apparently come in contact with a dilution of less than 1 : 400 or 1 : 500. The majority of hookworms found alive at autopsy were removed from the mucosa at levels 1/3 to 1/2 the intestinal length from the pyloric valve.

Heavier concentrations of bephenium were required to kill *Toxocara* and it is probable that those worms fairly close to the pyloric valve at the time the animals were dosed were the ones killed, for at that level the drug was apparently present in a stronger concentration.

Side effects. The only side effects noticed in these animals were nausea and vomiting. Of the 54 animals used in this series (50 for *Ancylostoma*, 3 for *Trichuris* orally, and 1 for *Physaloptera*), only 9 had nausea and vomiting. Seven of these received dosages of 50 mg/kg or over. At least 2 of the animals that vomited after dosing were known to have vomited for an unknown cause the week prior to treatment. Of the 3 dogs given 50 mg/kg daily for 5 or 6 days, only 1 vomited and that was a single time between the second and third doses. Although vomiting may occur occasionally, it is rare in those animals receiving an effective dose of 20 to 25 mg/kg or less.

DISCUSSION

For a number of years tetrachlorethylene, n-butyl chloride and hexylresorcinol have been used in the treatment of hookworm infections in dogs and cats. The first two require preliminary fasting and a purge is given several hours after treatment. The first two are given in dosages of up to 5 ml and the third up to five 0.2-gm capsules for larger dogs. Single doses of these compounds generally give a worm removal rate of 80 to 90%.

Recently McCowen et al (1957) reported on the use of dithiazanine for the removal of several worm infections in dogs and cats. This compound was administered at the rate of 10 mg/kg daily or twice daily for 5 to 7 days, or, in some cases, for as long as 3 months. Complete elimination of hookworms was obtained.

Single doses of the various bephenium salts, even as low as 10 mg/kg, gave a hookworm elimination rate of 97% in 50 naturally infected dogs and cats. And in 9 of the 11 animals not cleared, only a single hookworm remained. A single dose of 20 to 25 mg/kg is recommended for routine treatment and should remove 99 to 100% of the hookworms. There is a possibility that dogs and cats in more southern areas will harbor heavier infections and may require single doses of 50 mg/kg or a repeated dose of 20 to 25 mg/kg.

SUMMARY

1. Various bephenium salts given to naturally infected dogs and cats in single doses of over 20 mg/kg gave 99.4% clearance and single doses of 20 mg/kg or less gave 94.1% clearance of *Ancylostoma caninum*.

2. About two-thirds of the roundworms, *Toxocara*, were killed by single doses of 50 mg/kg or over, but only about one-fourth were killed with lower doses. These counpounds were only slightly effective against *Trichuris vulpis* and ineffective against 3 species of tapeworms.

3. One *Physaloptera* infection was treated and 50% of the worms were killed with a single dose of 50 mg/kg of bephenium chloride.

4. In vitro studies were made of the action of bephenium chloride on *Ancylostoma*, *Toxocara* and *Trichuris*.

5. The only side effects noted were occasional nausea and vomiting.

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BEPHENIUM, A NEW DRUG ACTIVE AGAINST HUMAN HOOKWORM

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Hookworm (*Necator americanus*) infections in mental patients constitute a severe problem in many areas because of their severity and the difficulty of effective treatment. The drugs in common use against hookworms ordinarily give poor results with such patients. For a number of years, a search for better drugs has been carried on at this hospital.

Recently a preliminary report indicated that bephenium, the generic name of a new series of compounds, i.e., the 2-phenoxyethylammonium salts, was active against certain nematodes, especially the mucosa-dwelling species, parasitic in the gastrointestinal tract of mammals (Copp, Standen, et al, 1958). Burrows (1958) obtained 99.4% clearance of *Ancylostoma caninum* in dogs and cats using 2-phenoxyethylammonium salts in single doses greater than 20 mgm/kg. Single doses of 20 mgm/kg or less produced results almost as good.

The chemical name of the drug used in the current experiments is N-benzyl-N, N-dimethyl-N-2-phenoxyethylammonium chloride. It was furnished to us by Burroughs-Wellcome as B.W.300C55 with the following pharmacological information:

The drug apparently is not well absorbed from the intestine since in mice the LD-50 for the chloride, intraperitoneal, is 72 mgm/kg but about 1,000 mgm/kg when the drug is given orally. Rats dosed orally with 500 mgm/kg of the embonate for 9 weeks showed no hematological changes or histological evidence of toxicity.

Monkeys given 317 mgm/kg of the chloride and 500 mgm/kg of the hydroxynaphthoate and embonate 5 days a week for 5 weeks showed no significant abnormality in the tissues.

When given intravenously the effects of the drug are markedly increased. It produces brief falls in the blood pressure of cats or dogs.

Small doses, 0.25 mgm/kg, prolong the action of epinephrine; large doses block the pressor effect. The drug produces some ganglionic blockade and causes a bradycardia. Intense mydriasis was observed in cats, and dogs exhibited salivation and diarrhea. Possible central action is indicated by emetic effects in dogs and respiratory failure. In lethal ranges this appears to be the cause of death.

In view of the various studies it appeared justified to test the chloride salt against human hookworm at doses of 20 mgm/kg given orally.

The patients were white female inmates of a mental hospital who were selected because they were known to have moderate to heavy hookworm infections. The density of the worm eggs in the feces was determined by the Stoll method, 2 deter-

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minations being done immediately before the drug was given and 2 during certain weeks after the treatment. There were no pretreatment nor posttreatment procedures, such as purges, etc.

The weight of the patients averaged about 55 kilograms (121 lbs.). The bephenium chloride salt was used in capsules containing 400 mgm each. The base was 88.03% of the salt. The drug was administered as a single 1.2-g dose of the salt, equivalent to 1.056 g of base, before breakfast on either 1 or 5 days. It was acceptable to the patients; no nausea or vomiting occurred. There were no toxic manifestations related to the drug. The results are shown in table I.

TABLE I—*Results showing effect on hookworm infections of 2 regimens of bephenium chloride (500C55).*

Dose of base	Single 1.056 grams		1.056 grams d × 5 d	
No. Patients	14		6	
	Average eggs per gram feces			
	Range	Average	Range	Average
Before Rx:	700–50,200	7,700	7,000–79,300	24,700
After Rx:	Percentage reduction		Percentage reduction	
1 week	0–100	27	77– 99	91
2 weeks	—	—	48–100	87
3 weeks	0–100	49	41– 99	85
4 weeks	—	—	78– 99	86
15 weeks	0–100	54	50–100	79

The single 1.2-g dose of drug removed about half of the hookworms as determined 3 and 15 weeks after the treatment. While these were better results than ordinarily obtained in this hospital with the commonly used drugs, a 5-day regimen totaling 6 g was instituted to determine if a better effect could be obtained. The six patients receiving this treatment were examined twice weekly for 4 weeks after therapy and again in the 15th week. The final determinations indicated that about 79% of the worms were removed.

The 14 patients receiving the single 1.056-g dose also were infected with *Trichuris trichiura*. The examination 15 weeks later revealed that 1 infection had been eliminated and that the overall reduction in the egg count was 14%.

This drug shows promise of being more effective against hookworm infections than any compound now commonly used. It is planned to try other salts of the compound and other regimens.

SUMMARY

Bephenium chloride, representing a new series of compounds (the 2-phenoxyethylammonium salts), was found to exert significant effect against human hookworm. A single dose of 1.2 g, equivalent to 1.0564 g base, eliminated about half the worms in 14 patients. Six grams, 5.280 g base, given over 5 days to 6 patients reduced the hookworms by 79%.

The single dose regimen reduced the *Trichuris trichiura* infections in the 14 patients by 14%.

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STUDIES ON THE DEVELOPMENT OF *DIROFILARIA TENUIS* CHANDLER 1942*

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Chandler (1942) reported and described the female of *Dirofilaria tenuis* from the subcutaneous connective tissue of a raccoon from eastern Texas and McIntosh (1954) described the male from a raccoon trapped on Longboat Key, Sarasota County, Florida.

Mosquitoes were first reported to transmit a dirofilariid (cited as *Filaria immitis*) by Grassi and Noe (1900) who observed its development in the malpighian tubules. Fleas also have been incriminated as vectors of *Dirofilaria immitis*. However Newton and Wright (1956) demonstrated the presence of 2 species of filarial worms in dogs in the United States, and Newton (1957) transmitted that *D. immitis* from dog to dog by *Anopheles quadrimaculatus*. The second worm discovered by Newton and Wright was a *Dipetalonema* from the subcutaneous connective tissue which would not develop in *Anopheles quadrimaculatus*. Other dirofilariids have been reported to develop in mosquitoes, i.e., *D. repens* by Raghaven et al (1952) and Webber and Hawking (1955), *D. scapiceps* by Highby (1938, 1943), *D. spinosa* by Highby (1939), and *D. aethiops* by Webber (1955). Development of *D. tenuis* from *Procyon lotor elucus* Bangs to infectivity in *Aedes taeniorhynchus* was reported by Pistey (1956) and details are submitted here.

MATERIALS AND METHODS

Wet slide mounts plus dry smears made with blood obtained from ear snips, and xenodiagnosis, were utilized to locate microfilariae in experimental animals. Methylene Blue 1:1000 was added to Knott (1939) technique concentrates to stain microfilariae, and 5% formalin was added when preservation for long periods was desired (Price, personal communication). First- and second-stage larvae were fixed on a slide with a drop of 2% formalin or Bles' (1905) fixative after being dissected out of the mosquito according to Carpenter et al (1946). The formalin-salt mixture was drawn up with filter paper and 2% formalin again added until only formalin remained. Larvae were stained with a drop of Taft's (1950) stain. Staining was complete in 1 to 3 minutes. The stain was then drawn off with filter paper, larvae were washed with 2% formalin and covered with a cover slip ringed with paraffin. Infective larvae were treated similarly after removal by the method of Phillips (1939).

Sixteen species of mosquitoes were used in the study. The *Culex pipiens* and *Aedes triseriatus* were from Connecticut, *Aedes aegypti* and *Anopheles quadrimaculatus* were laboratory-reared, and the remainder of the mosquitoes tested were wild caught in Florida.

Aedes taeniorhynchus and *A. sollicitans* were trapped on Longboat Key, at Punta Rassa, Gibsonton, and Lake Placid, Florida. Salt marshes north of Gibsonton, Florida, on U. S. route #41 produced *A. sollicitans* adults in June and early July, and *A. taeniorhynchus* in late July and early August. *Aedes atlanticus-tormentor, A. infirmatus, A. fulvus pallens, Psorophora ferox,* and *P. ciliata* were collected in Highlands Hammock State Park just north of Sebring, Florida. The majority of *P. confinnis* were collected around Lake Placid, Punta Rassa, and near the Archbold Biological Station. *Culex nigripalpus* and other species of *Culex* were ob-

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tained at the Archbold Biological Station, Hillsborough County State Park, and on Longboat Key. *Wyeomia vanduzeei* and *W. mitchelii* were collected on Longboat Key.

Wild adult females were trapped in a portable net placed over a 30" × 18" × 20" cage containing an infected raccoon. A damp cloth was placed over one corner to attract blood-fed mosquitoes and one corner was left open to permit the mosquitoes to enter the trap. Blood-fed mosquitoes were aspirated, counted, identified, and placed in separate quart paper containers screened at both ends with nylon netting. At the laboratory the containers were placed end down in a pan containing an inch of water to maintain proper humidity, and supported on 4 wooden blocks immersed in 2-ethylhexanediol-1,3 to prevent ants from destroying the mosquitoes. Mosquitoes were fed daily with a cotton pad saturated with honey water placed on top of the container. Feeding was stopped on the 9th day to permit infective larvae to accumulate in the proboscis. A maximum of 100 mosquitoes could be maintained in each container on which all data for each batch were recorded.

Noninfected raccoon recipients were exposed to bites of experimentally infected mosquitoes, and dogs were inoculated subcutaneously with known numbers of infective larvae which had been dissected out of the mosquito's proboscis.

RESULTS

A series of experiments was carried out, designed primarily to screen potential vectors. Exposed mosquitoes were identified and checked periodically to observe developing larvae. Table I lists the mosquitoes tested. In many instances the low number of individuals reported in no way reflects their incidence in the area as individuals of some species would not feed readily.

Culex pipiens, Aedes triseriatus, and *Psorophora ciliata* refused to feed after repeated attempts to induce them to do so. No development at all occurred in *Culex* spp., *Culex nigripalpis, Aedes aegypti, A. atlanticus-tormentor, A. fulvus pallens, Wyeomia vanduzeei,* and *W. mitchelii.* None of the species which survived suffered any apparent effects from the infected blood meal, and no visible larvae, or larval remains, were found after the 5th day in any individual of this group.

TABLE I.—*Mosquitoes investigated and maximum development observed.*

Mosquito	Total No. Mosq. Fed on Infect. Blood Meal	Max. Stage of Development Seen
Aedes aegypti (Linnaeus)	264	Prelarva
Aedes atlanticus-tormentor	134	Prelarva
Aedes fulvus pallens (Ross)	4	Prelarva
Aedes infirmatus (Dyar et Knab)	10	9th Day
Aedes sollicitans (Walker)	7	Infective
Aedes taeniorhynchus (Wiedmann)	2007	Infective
Aedes triseriatus (Say)	0*	.....
Anopheles quadrimaculatus (Say)	124	Infective
Culex nigripalpus (Theobald)	95	Prelarva
Culex pipiens (Linnaeus)	0*	.....
Culex spp. unidentified	283	Prelarva
Psorophora ciliata (Fabricius)	0*	.....
Psorophora confinnis (Lynch-Arribalzaga)	30	Infective
Psorophora ferox (Humboldt)	46	7th Day
Wyeomyia mitchelii (Theobald)	11	Prelarva
Wyeomyia vanduzeei (Dyer et Knab)	19	Prelarva

* Refused to feed on the infected raccoon.

Advanced normal larval stages were found in all *Aedes sollicitans* examined, but only 1 specimen harbored active infective larvae. One specimen of *Psorophora confinnis* examined displayed active third-stage larvae in the proboscis and haemocoel. Others were negative. One *P. ferox* had second-stage larvae in the tubules on the 7th day. Two specimens of *Aedes infirmatus* had viable second-stage larvae in the tubules on the 9th day.

Complete development occurred readily in *Anopheles quadrimaculatus* and *Aedes taeniorhynchus,* but the latter proved to be the most highly acceptable species for development of *D. tenuis.* Its numbers and efficiency at maintaining the parasite

indicates its roles as the natural vector of this helminth on Longboat Key, a 12 × ¼ mile key near Sarasota, Florida. (The local mosquito control commission eliminated essentially all but 2 species of mosquitoes by cutting the key with drainage ditches which were constantly flushed by the tide. Studies of earlier mosquito records of the area have proved the effectiveness of the control of fresh water breeders; however *Aedes taeniorhynchus* and *A. sollicitans* continued to breed in abundance. Five of 11 raccoons trapped on the key were infected with *D. tenuis*.)

Development of Larvae in Mosquitoes

General Development

Larval stages are described as found in *A. taeniorhynchus*. Temperature and relative humidity fluctuations were those that occurred near Sarasota, and Lake Placid, Florida, during June, July, August, and September of 1954, 1955, and 1956.

In general, development of *D. tenuis* larvae closely parallels the development of *D. immitis* and other filarial worms which develop in the malpighian tubules of mosquitoes. After ingestion by the mosquito, prelarvae migrate from the gut directly to the tubules where development is somewhat asynchronous. The larvae undergo 2 molts in the arthropod. After the second molt, which generally occurs in the tubules, access to the haemocoel is gained by active penetration of the malpighian tubules, or via a migration from tubules to ventriculus. Larvae perforate the wall of the ventriculus and enter the haemocoel (Plate IV, Fig. 2). The migratory route eventually terminate in the proboscis with infective or third-stage larvae being found there 10 to 12 days after the infected blood meal (Plate IV, Fig. 1). In heavy infections, numerous third-stage larvae can be located in the head capsule as well as the labium. At 17 days, larvae in the proboscis lose their activity, and possibly their infective potential.

First and Second Days

Microfilariae (now termed prelarvae) ingested by the mosquito undergo no visible morphological changes. There is, however, an active migration from the distended midgut to the malpighian tubules which is completed by the 3rd day after the infected blood meal, and prelarvae that remain in the gut, although alive, appear sluggish.

No development can be observed of larvae which remain in the midgut. They eventually die, and are either completely digested, or lost in the feces.

Mosquitoes which have taken a large infected blood meal occasionally defecate a portion of the meal, resulting in a considerable loss of blood and prelarvae. This phenomenon has been noted by Reid (1935) and Kartman (1953a, 1953b).

Occasional larvae undergo pigmental encapsulation (Bahr, 1912; Manson and Bahr, 1921; Fülleborn, 1929) or "brown degeneration" (Noe 1901) when in the tubules. The phenomenon (Plate IV, Fig. 3, 4) is not unique, for references to "chitinous" capsule-formation around the parasite in mosquitoes are numerous in the literature. They are blackish-brown and opaque and remain in this state up to 15 days after encapsulation. It was found only occasionally. Encapsulation beyond the prelarval stage was rarely encountered, and was confined to the caudal filament of developing first-stage larvae.

Third Day

Early in the 3rd day gross changes occur and vitally stained preparations can be utilized. Fixation and then staining with Taft's stain provided the most favorable results. The larva decreases in length and increases in diameter.

Three types of nuclei can be distinguished. The first type is large, elongated, and oriented superficially at an angle to the long axis of the body. These stain deeply and clearly and probably correspond to the muscle cells of hypodermal nuclei reported by Rodenwaldt (1933). They are visible throughout the early first-stage larvae and begin to disappear at approximately the 5th day of development (Plate I, Fig. 1).

The second type is more spherical and stains a bright blue with the Taft preparation. Most characteristic are 2 distinct pairs of cells resembling eyes which lie in 2 distinct levels in the anterior part of the organism (Plate I, Fig. 1).

The third type of nucleus, found in the large excretory cell, stains deeply, and possesses a distinct nucleolus. Anterior to this cell the excretory pore bladder can be seen as a pink staining ring. The nerve ring is distinguished behind the 4 anterior cells as a group of small cells along a localized periphery of the larva. Posteriorly, a group of large cells with spherical nuclei begins to appear in a linear fashion, and eventually divides to form the intestine. The rectal cells orient themselves in a triangular pattern ventral to which the rectal plug begins to erupt as a small papillary evagination of the cuticle.

Three caudal nuclei stain deeply and become delimited from the body of the larva. This appears to be an indication of the first cuticular ecdysis.

Fourth Day

The first-stage larva on the 4th day in no way resembles the prelarva. Compressed linearly between 110 to 123.5 microns, it expands from 6 to 7 microns to a maximum of 18 microns in width, and possesses, with the exception of the caudal filament, every appearance of a sausage stage (Plate I, Fig. 2). Anteriorly, 4 papilla-like structures appear to be a wrinkling of the cuticle, probably an accommodation for the sudden change in the larval size.

The superficial muscle cells, or hypodermal nuclei, persist in the anterior region immediately posterior to the cuticular wrinkles. The nerve ring is faintly visible as a group of small cells running diagonally across the linear axis of the larva just posterior to the cuticular wrinkles. The excretory cell is readily visible, and is connected to the anterior red-staining excretory pore by a very short, wide canal terminating in a pouch-like structure immediately dorsal to the pore itself. The pore exists on approximately the same plane as that of the rectal plug. Approximately 13 to 14 microns behind the nucleus of the excretory cell a small group of cells of 2 sizes begins to divide rapidly. These eventualy form the junction of the gandular portion of the esophagus and the intestine. The larger of these cells helps form the intestine. The smaller ones form the esophageal portion of the junction. Posteriorly a slow orientation of large cells containing large spherical nuclei takes place along the axis of the larva (Plate I, Fig. 3).

The rectal plug enlarges considerably and small cells forming the rectal primordia become indistinct while the rectal region becomes distinctly demarcated from other Anlagen in the area.

The caudal filament begins to curl, and in rare instances turns brown and opaque. Caudal nuclei become only faintly visible as their staining properties decrease steadily.

Fifth to Seventh Days

These 72 hours are characterized by prolific activity of the cellular Anlagen of the cuticle, nerve ring, esophagus, intestine, and rectum. Cells of the nerve ring are more numerous, and esophageal cells begin to proliferate from the center both anteriorly and posteriorly. The stoma is not open. A group of from 6 to 10 large cells with large spherical nuclei line up most conspicuously in the posterior half of the larva, parallel to the linear axis of the worm. These cells begin the formation of the intestine which becomes separated from the small oblong cells that lay down the new cuticle. Separation is on approximately the 6th day. Separation of the intestine from the hypodermal cells commences posteriorly and proceeds anteriorly. At the end of the 5th day the union of intestine and rectum begins to occur, and the two remain as distinctly visible structures up to the infective stage. Between the 5th and 6th days, the Anlagen of the reproductive system appear anterior to the rectal plug as a small group of compact, oblong cells with spherical nuclei. On the 6th day the rectum is found to be anchored to the opposite wall of the larva (Plate II, Fig. 2; Plate IV, Fig. 6), and the lumen of the rectum appears in the center of the rectal cells.

During the 6th and 7th days, the separation of the gut from the hypodermal cells, and the formation of the pseudocoel is completed although junctions between the esophagus and intestine are not clear.

The lumen of the rectum has become larger by the 7th day, and the excretory pore and cell appear less distinct. The cuticle to be shed projects from the stoma and remains posteriorly as the tail filament. Caudal nuclei can no longer be distinguished. The junction of esophagus and intestine is distinctly visible as a group of cells of 2 sizes. In 72 hours the larva has increased in dimension from approximately 139 by 16 microns to 214 by 18 microns.

Eighth and Ninth Day

Most significant by the 8th day is the development of the lumen of the alimentary tract. Esophagus, intestine, and rectum are recognized easily for they are completely separated from the wall of the larva; the pseudocoel is complete. The lumen of the rectum enlarges but does not join the lumen of the intestine. The lumen of the intestine begins in 2 areas, i.e., one area posterior to the esophagus, the other anterior to the rectal-intestinal junction. The lumen in each area increases in size both anteriorly and posteriorly until they eventually meet in the center of the intestine. The lumen of the esophagus appears as a faint line along the center of its entire length. It increases its diameter slightly by the 8th day, and becomes continuous with the lumen of the intestine. The stoma is not connected to the esophagus until the first molt which usually occurs between the 7th and 8th day. The junction between stoma and esophagus is complete after the first cuticular ecdysis. Actual molting was always observed to occur in an anterior-posterior direction. Although the rectal plug is part of the cuticle, it persists after the first molt and measures approximately 18 microns along its base.

The nerve ring is distinct and takes on a fibrous appearance. The excretory pore is now only faintly visible. The second-stage larva becomes active, reacts favorably to Taft's stain, and now measures approximately 610 microns by 35 microns (Plate II, Fig. 3). Malpighian tubules show evidence of this increase in size for they

become distended, particularly at their terminal extremities where larvae accumulate and become active. Distention of the tubules appears to be mechanical.

Activity increases steadily as the larva wanders anteriorly through the tubules. The larva increases in length and lays down new cuticle in preparation for the second molt which usually occurs in the tubules between the 9th and 10th day (Plate III, Fig. 1). In cases where large numbers of microfilariae were ingested, molting occurred occasionally in the haemocoel.

The lumen of the intestine is completed and joined with the lumen of the rectum. The rectal canal leading to the anus is completed and becomes structurally functional after the second molt. The rectal plug is lost with the cuticle.

Tenth and Eleventh Day

Following the second molt, the infective larvae migrate into the haemocoel via either the malpighian tubules or the ventriculus. They migrate anteriorly through the haemocoel following very closely, the path of the digestive tract through the thorax and into the head capsule, from which they enter directly into the haemocoel of the proboscis. Dutton (1901) reported that infective larvae of *W. bancrofti* ruptured a thin membrane at the junction of the labium and the labella (Dutton's membrane). The proboscis is structurally impervious to infective larvae, consequently they exit only at this point (Plate IV, Fig. 1).

Larvae recovered from the proboscis and head capsule can be divided roughly into 2 length groups, one measuring 781 to 994 microns (average 905.6 microns), the other measuring 1001.1 to 1157.3 microns (average 1056.1 microns). The width of larvae of both groups is essentially the same, measuring from 21.58 to 28.22 microns (average 23.9 microns). Although sex at this stage is indistinguishable, it is possible that individuals of each size group indicate a sexual distinction by size.

The nerve ring is distinct and fibrous in appearance (Plate III, Fig. 2), and the stoma is a short cuticular tube leading to the lumen of the esophagus. The gut is completely open from stoma to anus. No rectal plug remains, and the lumen of the intestine is larger than the thickness of its wall (Plate III, Fig. 3).

The supposed genital Anlage is a group of 6 to 8 cells half way between the esophageal-intestinal junction, and the rectum (Plate III, Fig. 2). The early formation of the vagina and vulva can be seen in some of the larvae.

The posterior end of the larva terminates bluntly and like the adult, tends to curl dorsally. No papillae are visible. Cuticular striations present on the microfilariae and adults are again visible (Plate IV, Fig. 5).

Experimental Infection

Two 3-week-old raccoon cubs, a female and a male born in captivity to a negative female, were exposed to bites of infective mosquitoes on August 30, 1955 and September 3, 1955, respectively. One *A. taeniorhynchus* fed on the female; 3 fed on the male. The raccoons were maintained in pens in Bridgeport, Connecticut, and checked monthly for microfilariae. Both raccoons were sacrificed on November 24, 1956, approximately 14 months after the experimental exposure. Concentration techniques produced no microfilariae in either recipient. The female on necropsy was negative. Two females 9.5 cm and 10.3 cm long lacking microfilariae, and 2 males 4.5 cm and 4.4 cm long, of *D. tenuis* were recovered from the subcutaneous connective tissue of the male raccoon.

Two 3- and 4-month old females (#3 and #4) caught wild in Englewood, Florida, were also exposed to infective mosquitoes. Raccoon #3 was bitten by 2 infective mosquitoes on July 6, 1956, and Raccoon #4, first anesthetized with Nembutal, was bitten by 9 infective mosquitoes on August 14, 1956. Both raccoons were sacrificed on March 21–22, 1957. Concentration techniques produced no microfilariae in either recipient. One 8.6-cm long female without microfilariae was recovered from the subcutaneous connective tissue of raccoon #4.

Four female mongrel pups were inoculated with known amounts of infective larvae dissected out of the proboscis and administered with a saline vehicle under the skin of the hip. The dogs received 6, 9, 25, and 44 larvae respectively. No microfilariae were recovered from the dogs, and necropsy 106–109 days after inoculation revealed no worms and no gross indication of tissue reaction at the site of inoculation.

Site of Infection in the Raccoon

The adult male raccoon, trapped on Longboat Key, Sarasota County, Florida, during the summer of 1954 and used as a source of microfilariae for mosquito transmission studies (Pistey, 1957) yielded on necropsy 37 females and 25 males of *Dirofilaria tenuis* from the subcutaneous connective tissue. The worms were rather evenly distributed along the perimeter of the body, as well as under the skin of all 4 legs, and were found over both masseter muscles, the parietal region of the head, and the lateral areas of the neck.

The raccoon apparently suffered no ill effects from this heavy infection (1,000+ microfilariae per 20 cmm at 4:00 P.M.). Weight was maintained and even increased in captivity. Pelage was normal, and no local or general deciduation of hair could be detected.

Plate IV, Figure 7, illustrates the position of the worm in a thin capsule between the adipose and dense subdermal reticular connective tissue in a section taken from the shoulder. There is a very slight and diffuse infiltration of polymorphonuclear leukocytes. A vein in the immediate vicinity of the worm showed a slight perivascular cuffing with lymphocytes and polymorphonuclear leukocytes. No pathologic alterations of the hair follicle could be detected. In general, the worm appears to be very compatible with its host, the reaction observed being very slight and generally considered negligible.

Between the coils of the worm is found a coagulated serum-like material. The source or purpose of this material is not known. It is possible that this substance acts as a medium into which microfilariae are released and from which they eventually migrate through the tissues into the vascular system.

DISCUSSION

The pattern of normal development of *D. tenuis* in the mosquito is similar to *D. immitis* and *D. repens*. All 3 species develop in the malpighian tubules. In addition to rupturing the tubules, larvae of *D. tenuis* migrate back into the ventriculus, penetrate its wall, and enter the haemocoel. This path of migration has not been reported for the other 2 species. Migration was always in a posterior to anterior direction, and the erratic migration of larvae in the haemocoel as reported by Hu and Yen (1933), Pratt and Newton (1946), and Rosen (1955) for *W. bancrofti* was absent in mosquitoes infected with *D. tenuis*.

Kartman (1953b) reported that microfilariae fed to mosquitoes in a sugar solution were found in the diverticula. The diverticula were free of microfilariae (prelarvae) in all cases examined in my study.

Kotcher (1941) working with frog filariae, and Scott et al (1951) working with *Litomosoides carinii* reported that microfilariae were not first-stage larvae because they did not molt before they arrived in the circulatory system. Augustine (1937) and Kershaw (1948) contended that the first molt of a filarial worm occurred in the uterus of the female, i. e., microfilariae were the first-stage larvae and the first sausage stage would then be second-stage larvae. Larvae of *D. tenuis* undergo 2 definite molts in the mosquito. The number of molts occurring in the raccoon is not known, yet on the basis of reports for other nematodes, 2 additional molts could be anticipated in the definitive host. Larvae in the mosquitoes which were morphologically identical to the microfilariae in the blood were called prelarvae. At the onset of visible changes in morphology they were termed the "first-stage larvae" until they molted. After the molt they became the "second-stage larva," etc. The majority of nematodes have 3 larval stages before they enter the definitive host. It is unlikely that *D. tenuis* follows a different pattern; consequently the reports of Kotcher (1941) and Scott et al (1951) seem acceptable in this regard.

Neither *D. immitis* nor *D. repens* possesses cuticular striations present on both the microfilaria and infective larva of *D. tenuis*. The microfilaria of *D. tenuis* is longer than these 2 species by approximately 120–130 microns. The literature gives no reference to cuticular wrinkles on *D. immitis* or *D. repens* larvae. Cuticular wrinkles occur early in the development of *D. tenuis* in the mosquito.

All 3 species develop to infectivity in 10 to 12 days in suitable hosts. Webber (1955) reported, however, that 14 to 16 days were necessary for development of *D. repens* in a poor host. Sufficient quantities of *Psorophora ferox* and *Aedes infirmatus* (poor hosts) were not available effectively to determine if a longer period was necessary for development in these species.

The long prepatent period found in the experimental infection of the raccoons was unexpected. The worms recovered, however, were probably capable of producing microfilariae had they copulated. At necropsy they were not found near each other. The prepatent period may be less than seven months under optimum conditions.

SUMMARY

Sixteen species of mosquitoes were tested for their ability to maintain to infectivity the larval stages of *Dirofilaria tenuis*. Development occurred readily in the malpighian tubules of *Aedes taeniorhynchus* and *Anopheles quadrimaculatus*. Development occurred in 7–8 days for first stage larvae and approximately 2 days for second stage larvae, i. e., the first molt occurred in 7–8 days, and the second molt 9–10 days after the infected meal. Infective larvae were found in the proboscis 10–12 days after feeding on an infected raccoon. Migration to the proboscis via the haemocoel after the tubules and ventriculus had been actively penetrated was observed. *Aedes sollicitans* and *Psorophora confinnis* could probably be considered potential vectors as infective stages were recovered from these species. *A. taeniorhynchus* is the natural vector of this nematode on Longboat Key, Florida.

Two of 4 experimentally exposed raccoons developed the infection; however, no microfilariae were recovered from the blood of any of these animals. The adults

of *D. tenuis* were recovered 7 and 14 months after the experimental exposures. No adults or microfilariae of *D. tenuis* were recovered from 4 puppies inoculated with known numbers of infective larvae.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS IN FIGURES

a.—anus; a. p.—anal pore; c. f.—caudal filament; cut. I—cuticle of first stage larva; cut. II—cuticle of second stage larva; cut. III—cuticle of third stage larva; c. nuc.—cephalic nuclei; ex. b.—excretory bladder; ex. c.—excretory cell; ex. p.—excretory pore; e. i. j.—esophageal-intestinal junction; eso—esophagus; G—"G" cells; g. m.—genital mass; hyp. n.—hypodermal nuclei; int.—intestine; int. const.—intestinal constriction; int. lum.—intestinal lumen; n. r.—nerve ring; pseud.—pseudocoel; r.—rectum; r.i.j.—rectal-intestinal junction; r. lum.—lumen of rectum; r. m.—rectal muscle; r. p.—rectal plug; st.—stoma.

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RESEARCH NOTE

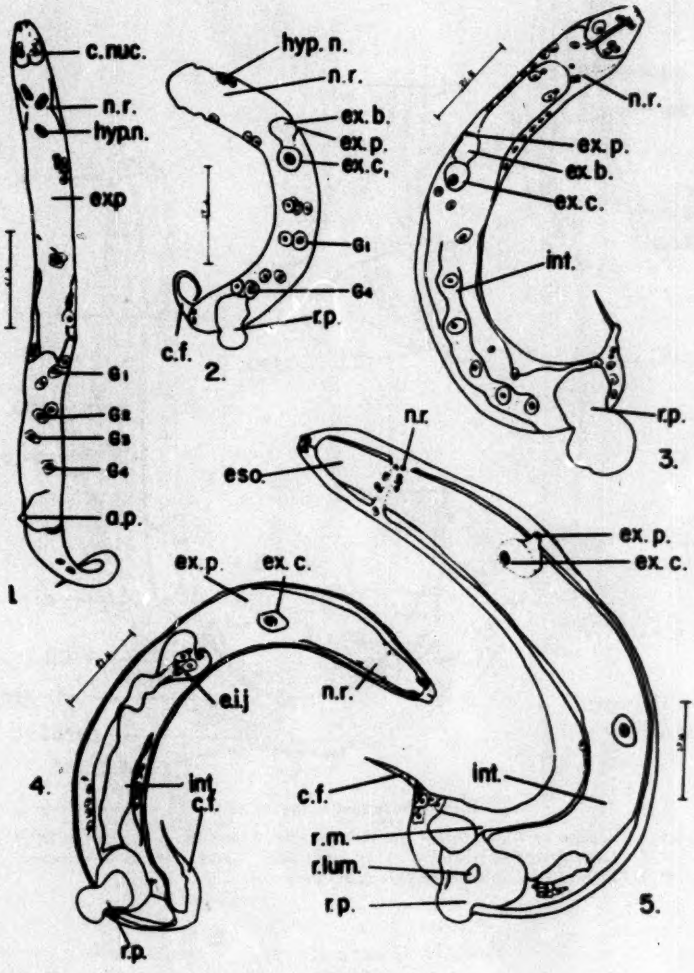
TROPICORBIS PHILIPPIANUS (DUNKER) A POTENTIAL INTERMEDIATE HOST OF *SCHISTOSOMA MANSONI* IN ECUADOR

The presence of potential intermediate hosts of *Schistosoma mansoni* living out of the endemic areas was first demonstrated by Cram et al (1945, Science **101**: 302) when they showed that *Tropicorbis havanensis* from Louisiana (U.S.A.) could be successfully infected with a Puerto Rican strain of the trematode. Recently, Barbosa & Barbosa (1958, Bol. Chil. Parasitol. **13**: 7) were able to infect *Tropicorbis chilensis* from Santiago, Chile, with a Brazilian strain of *S. mansoni*.

In the present report, preliminary results demonstrated that another *Tropicorbis* species, *T. philippianus* from Guayaquil, Ecuador, is susceptible to a Brazilian strain of *S. mansoni*. Seventeen laboratory-reared snails were each exposed to 20 miracidia concentrated from human feces in Belo Horizonte, Brazil. Thirty days after the exposure, 14 snails were alive. From these, one shed cercariae of *S. mansoni* 28 days after the exposure. The temperature of the water during the experiments ranged from 24 to 26° C.

Although schistosomiasis has never become established on the West coast of South America, malacological studies reveal that at least 2 species of *Tropicorbis* inhabiting these regions may be suitable for the development of the larval stages of *S. mansoni*.—F. S. BARBOSA, I. BARBOSA AND J. D. RODRIGUEZ, *Centro de Pesquisas Aggeu Magalhães, Recife, Brazil, and Instituto Nacional de Higiene, Guayaquil, Ecuador.*

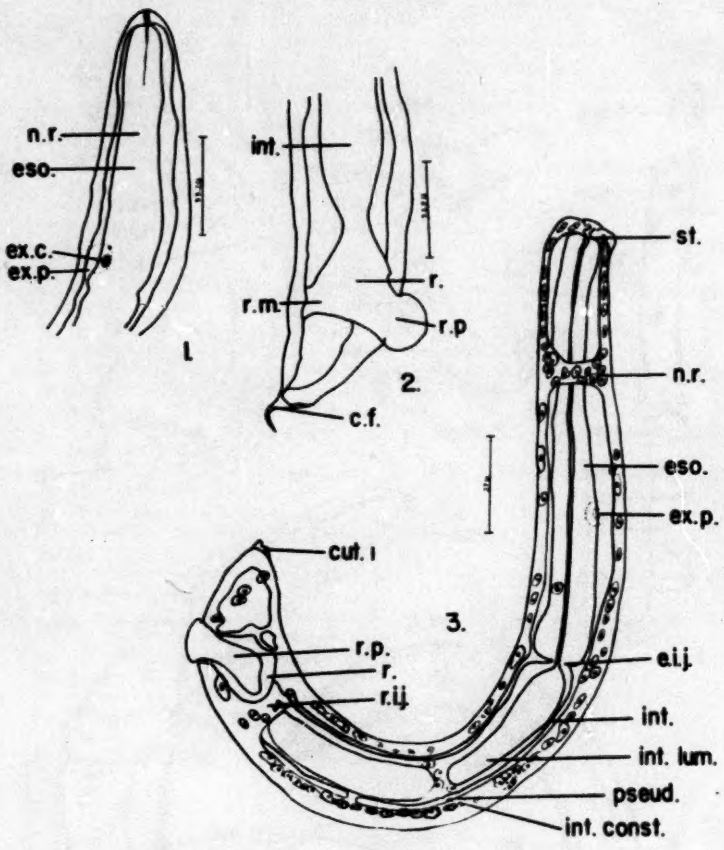
PLATE I



First-stage larva

FIGURE 1. First-stage larva after 3 days in *A. taeniorhynchus.*
FIGURE 2. First-stage larva after 4 days in *A. taeniorhynchus.*
FIGURE 3. First-stage larva after 5 days in *A. taeniorhynchus.*
FIGURE 4. First-stage larva after 7 days in *A. taeniorhynchus.*
FIGURE 5. First-stage larva after 6 days in *A. taeniorhynchus.*

PLATE II



First- and second-stage larvae

FIGURE 1. Lateral view of anterior end of larva after 7 days in *A. taeniorhynchus.*
FIGURE 2. Lateral view of posterior end of larva after 7 days in *A. taeniorhynchus.*
FIGURE 3. Lateral view of early second-stage larva.

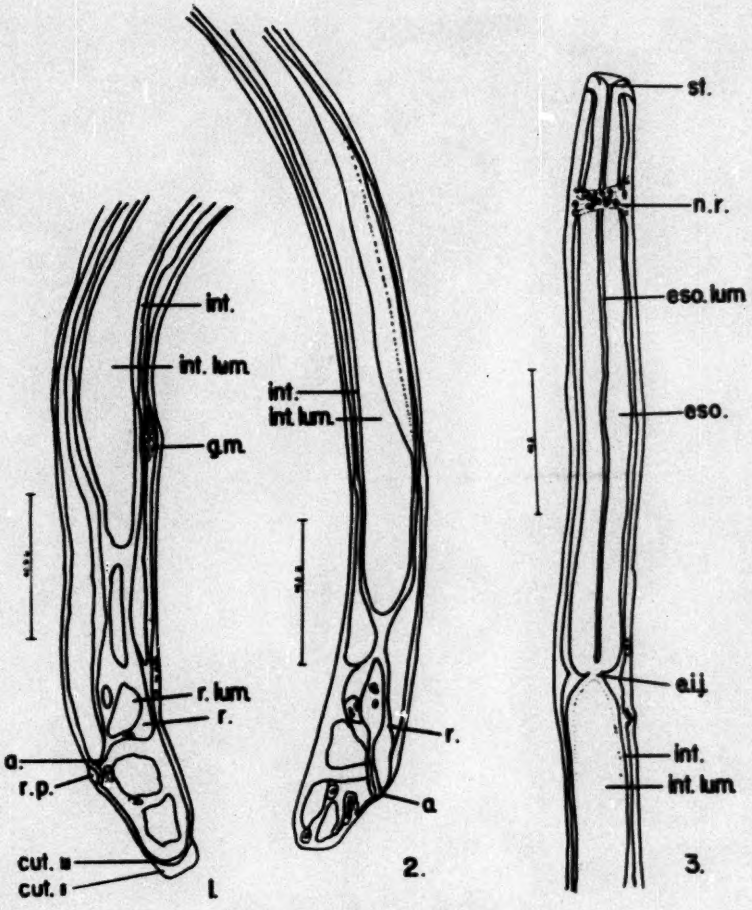
PLATE III

(see p. 625)

Third-stage larva

FIGURE 1. Lateral view of posterior end of late second-stage larva prior to molting.
FIGURE 2. Lateral view of posterior end of infective larva recovered from the proboscis of *A. taeniorhynchus.*
FIGURE 3. Lateral view of anterior end of infective larva recovered from the proboscis of *A. taeniorhynchus.*

PLATE III



(explanation on p. 624)

Explanation of Plate IV (p. 626)

FIGURE 1. Terminal portion of proboscis of *A. taeniorhynchus* illustrating site of extrusion of infective larvae. 150×.

FIGURE 2. Migration of an infective larva through the wall of the ventriculus of *A. taeniorhynchus*. 150×.

FIGURE 3. Pigmental encapsulation in the malpighian tubule showing difference between living and dead prelarvae. 430×.

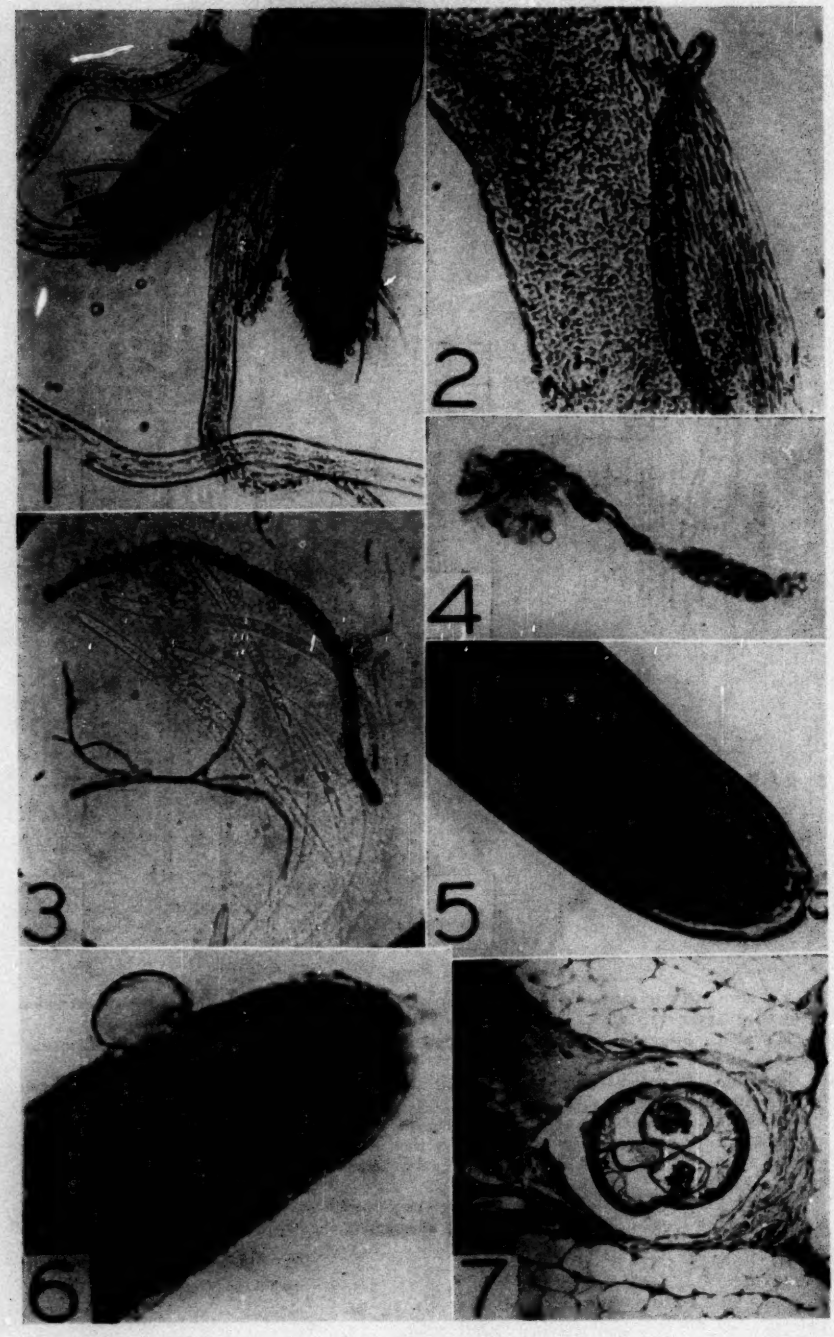
FIGURE 4. Pigmental encapsulation in the malpighian tubule. 100×.

FIGURE 5. Anterior end of infective larva showing cuticular striations. 1900×.

FIGURE 6. Posterior end of second-stage larva showing the rectal plug, rectum, and rectal muscle. 1900×.

FIGURE 7. Section of subcutaneous tissue cut perpendicular to the surface of the skin containing an adult female *Dirofilaria tenuis* and illustrating the slight capsule formed around the worm. Microfilariae are also visible. 90×.

PLATE IV



VARIATION IN THE NUMBER OF ORAL SPINES OF *PHAGICOLA LONGICOLLIS* KUNTZ AND CHANDLER, 1956, AND THE DESCRIPTION OF *P. INGLEI* N. SP. (TREMATODA: HETEROPHYIDAE)*

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During the course of investigations on a species of *Phagicola* from *Mugil cephalus* L., the Striped Mullet, and *M. curema* Cuvier & Valenciennes, the White Mullet, we have had the opportunity to examine numerous specimens of *Phagicola longicollis*. Through the courtesy of Allen McIntosh, Parasitologist, Animal Disease and Parasite Research Branch, U. S. Department of Agriculture, the type slide, U. S. National Museum Helminthological Collection No. 38169, was obtained. This slide contains a number of specimens of *P. longicollis* (Fig. 1), two *P. ascolonga* (Witenberg, 1929) Price, 1932, and several *Heterophyes heterophyes* (Siebold, 1852) Stiles & Hassall, 1900. *Phagicola longicollis* can be readily separated from the 2 specimens of *P. ascolonga* by the fact that in the former the uterus never extends posteriorly beyond the testes while in the latter the uterus always extends beyond the testes. Other differences include shape of body, structure of gonotyl, and posterior extent of the oral appendage.

We observed, on the type slide, some specimens (syntypes) of *P. longicollis* with 14, others with 15, and three with 16 oral spines in a single row. Some damaged specimens had less than 14 oral spines. The original description of this species by Kuntz and Chandler (1956) states: ". . . Mouth surrounded by single circle of 14, sometimes 15, spines, . . ." The late Dr. A. C. Chandler, in a personal communication (1957), stated: ". . . I counted the oral spines on several dozen specimens of *Phagicola longicollis* and found 14 in most, 15 in some, and thought I counted 16 in a few, but decided I had made an error. However, it is quite possible that there is a variation from 14 to 16 in this species, so some of the specimens on which you found 16 spines *may* belong to this species. . . ."

Dr. Chandler kindly sent us most of his specimens of *P. longicollis* and *P. ascolonga* for examination. Included in this material were specimens of a species of *Phagicola* that were obviously not *P. ascolonga*. Except for 1 specimen (Fig. 3) having a coronet of 17 oral spines in a single row and others having 16 oral spines they conform with the original description of *P. longicollis*.

We feel, therefore, from the observations stated above, the description of *P. longicollis* should be expanded to include a variation of from 14 to 17 oral spines.

Stunkard and Uzmann (1955) discuss the *Ascocotyle-Phagicola* complex. They conclude, "Decision on the taxonomic status of *Phagicola* should await more complete information, especially on the developmental stages of its members." Additional information may show that *Phagicola* is not a valid genus. However, it has

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** Contribution No. 13.

been the practice of some workers (Chandler, 1941; Kuntz and Chandler, 1956; Robinson, 1956) to describe several species under the generic name of *Phagicola*. They evidently separated *Phagicola* from *Ascocotyle* because the former had a single row or an incomplete second row of oral spines, body incompletely spined and vitellaria not extending forward beyond the level of the ovary, while the latter has 2 complete rows of oral spines, body completely spined and vitellaria extending forward anterior to the level of the ovary. We choose to follow the above practice of separating *Phagicola* from *Ascocotyle*, at least until the taxonomic status of *Phagicola* is accurately determined.

While examining the slides sent to us by Dr. Chandler one of us (RFH) observed 1 specimen of a species of *Phagicola* very closely related to *P. longa* (Fig. 2) and *P. longicollis*. It differs in a number of characteristics from either *P. longa* or *P. longicollis*. Because of this we considered it an undescribed species for which we propose the name of *P. inglei*. The name *inglei* is in honor of R. M. Ingle, Director of Research, Florida State Board of Conservation, in recognition of his foresight in establishing a marine parasite and disease program at our Laboratory.

Phagicola inglei n. sp.
(Figs. 4–10)
(All measurements in microns)

Host: Domestic dog.
Location: Small intestine.
Locality: Streets of Cairo, Egypt.
Date of Collection: December 8, 1953.
Holotype: U. S. Nat. Mus. Helm. Coll. No. 38390.

Diagnosis (Based on a single specimen): *Phagicola sensu stricto:* Body shaped like elongated sack, tapering gradually towards anterior end. Cuticle spiny, except for narrow band immediately posterior to oral sucker and extreme posterior part of body. Length 1132; maximum width 373. Anterior end of oral sucker with rounded lip. Single row of 19 spines surrounding mouth; spines approximately 17 to 22 long with oval bases and curved tips. Oral sucker 68 in diameter with elongated oral appendage about 227 long, extending 282 from anterior end of body or approximately 7/8 distance to pharynx. Pharynx 64 long by 55 wide, located approximately 1/3 distance from anterior end of body. Esophagus about 126 long. Ceca about twice diameter of esophagus, extending posteriorly beyond level of acetabulum where they are no longer visible (possibly the result of overclearing of specimen and occlusion by eggs and other internal structures). Acetabulum rounded, about 68 long by 72 wide; located about 2/3 distance from anterior end of body. Genital pore a transverse slit-like opening immediately anterior and median to acetabulum. Lobes of gonotyl not visible. Testes 108 to 120 long by 79 to 82 wide; side by side near posterior end of body. Ovary about 105 long by 88 wide; anterior to dextral testis. Seminal vesicle large, median, posterior to acetabulum. Seminal receptacle indistinct, anterior to testes and sinistral to ovary. Vitellaria follicular, 6 to 8 follicles on each side of body, lateral around outer border of testes; extending from behind testes to anterior border of ovary. Uterus occupying area from anterior border of testes and vitellaria to immediately behind acetabulum in 3 to 4 transverse loops. Eggs 25 to 28 long by 12 to 14 wide. Excretory vesicle not observed.

DISCUSSION

Stunkard and Uzmann (1955) have questioned the validity of the genus *Phagicola* Faust, 1920, and have discussed their view in detail. Price (1932, 1935) has retained *Phagicola* Faust, 1920, and maintains that this genus differs from *Ascocotyle* Looss, 1899 by possessing a single crown of oral spines as compared with 2 complete rows of oral spines in *Ascocotyle*. Stunkard and Uzmann (1955) have rightly questioned Price's (1935) reasoning. *Phagicola angrense* (Travassos, 1916) Price, 1932; *P. diminuta* (Stunkard and Haviland, 1924) Price, 1932; and *P. lageniformis* Chandler, 1941, all possess, in addition to a complete anterior row of crown spines, 2 accessory spines which are dorsally located behind the first row of

spines, and which definitely form an incomplete partial row. *P. nana* (Ransom, 1920) Price, 1932 also possesses an incomplete posterior dorsal row of crown spines, but instead of 2 there are 3 to 4 accessory spines. Stunkard and Haviland (1924) named the sub-genus *Parascocotyle* for *Ascocotyle* (*Parascocotyle*) *diminuta*. These authors were apparently unaware of Faust's (1920) genus *Phagicola*. The original description of *P. diminuta* made no mention of 2 accessory spines in the posterior crown. Stunkard and Uzmann (1955) later corrected this *lapsus*. Witenberg (1929) elevated *Parascocotyle* to generic status on the basis of the vitellaria extending anterior to the ovary in *Ascocotyle* species, and restricted behind the ovary in the former genus. Price (1932) clarified the error in the original description of the gonotyl of *P. pithecophagicola* and considered *Ascocotyle* (*Parascocotyle*) *diminuta* in the genus *Phagicola*. He not only synonymized *Parascocotyle* with *Phagicola*, but in 1935 considered *Metascocotyle witenbergi* Ciurea, 1933, type species, a synonym of *Phagicola longa* (Ransom, 1920) Price, 1932. *Metascocotyle* Ciurea, 1933, is definitely a synonym of *Phagicola* Faust, 1920. Ciurea (1933) was misled by Ransom's (1920) description of the gonotyl in *Ascocotyle longa* Ransom, 1920. Until life cycle studies prove differently, we prefer to retain the genera *Ascocotyle* Looss, 1899, *Phagicola* Faust, 1920, and *Parascocotyle* Stunkard and Haviland, 1924.

The following key will serve to separate these genera.

1. Vitellaria extending as far forward as acetabulum; with two complete rows of oral spines ..... *Ascocotyle, sensu stricto*
1.—Vitellaria extending forward only to ovary, never beyond; never with two complete rows of oral spines 2
2. With a single complete row of oral spines ..... *Phagicola, sensu stricto*
2.—With a single complete row of oral spines and an incomplete second row of from 2 to 4 accessory spines ..... *Parascocotyle, sensu stricto*

The name *Parascocotyle* Stunkard and Haviland, 1924 is available with *P. diminuta* Stunkard and Haviland, 1924 as type species, in spite of the fact that the authors' definition of the sub-genus was different from our views as presented in the key above. Since Stunkard and Uzmann (1955) have indicated the presence of accessory spines in the oral crown of *P. diminuta*, and we wish to group together the species with accessory spines and vitellaria not extending anteriorly past the ovary, the name *Parascocotyle* must be used by reason of priority. We have obtained specimens of *P. diminuta* by feeding the gills of naturally infected Floridian *Fundulus similisis* to hamsters.

According to the systematic scheme presented above, the following species would be included in the genus *Ascocotyle* Looss, 1899: *A. angeloi* Travassos, 1928, *A. coleostoma* (Looss, 1896) Looss, 1899 (type species); *A. filippei* Travassos, 1928; *A. intermedius* Srivastava, 1935; *A. leighi* Burton, 1956; *A. mcintoshi* Price, 1936; *A. megalocephala* Price, 1932; *A. puertoricensis* Price, 1932; and *A. tenuicollis* Price, 1935.

Under our system the following species would be included in the genus *Phagicola* Faust, 1920: *P. arnaldoi* (Travassos, 1929) Price, 1932; *P. ascolonga* (Witenberg, 1929) Price, 1932; *P. byrdi* Robinson, 1956; *P. italica* (Alessandrini, 1906) Price, 1932; *P. longa* (Ransom, 1920) Price, 1932; *P. longicollis* Kuntz and Chandler, 1956; *P. macrostomum* Robinson, 1956; *P. minuta* (Looss, 1899) Price, 1932; *P. piriforme* (Blanc and Hedin, 1913) Price, 1932; and *P. pithecophagicola* Faust, 1920 (type species).

The following species would be included in this system under the genus *Parascocotyle* Stunkard and Haviland, 1924: *P. angrense* (Travassos, 1916); *P. diminuta* Stunkard and Haviland, 1924 (type species); *P. lageniformis* (Chandler, 1941); and *P. nana* (Ransom, 1920).

Phagicola inglei appears to be most closely related with *Phagicola longa* (Ransom, 1920) Price, 1932. Witenberg (1929) and Martin (1953) have indicated that the oral appendage of *Phagicola ascolonga* and *Parascocotyle lageniformis* respectively, may extend to anterior margin of the pharynx or beyond. Although the significance of the relationship of the oral appendage with the pharynx is questionable, we would like to indicate that in the holotype of *P. inglei* and the syntypes of *P. longa*, of which the anterior end of the body appears to be equally relaxed, the oral appendage almost comes into contact with the pharynx in *P. inglei* as compared with oral appendage almost halfway to pharynx in the other species. The pharynx of *P. inglei* is located less than one-third of the body length from the anterior end while that of *P. longa* approaches the midbody. The vitellaria of *P. inglei* extend to the anterior border of the ovary while they are restricted behind the ovary of *P. longa*. *P. inglei* possesses 19 very heavy crown spines which are hooked at the tips as compared with 15–18 lighter, straight spines in the syntypes of *P. longa*. The esophagus of *P. inglei* is proportionately about 3 times longer, the eggs (Fig. 7) twice the length and diameter of those (Fig. 10) of the syntypes of *P. longa*. One of us (Hutton, 1957) reported *P. longa* from the mullet (*Mugil cephalus* and *M. curema*). Adult worms were obtained from laboratory-raised white rats fed on mullet. Since that time we have been able to obtain a species of *Phagicola* by feeding naturally infected mullet to hamsters, pelicans, chickens, mink, kittens, herons, and opossums reared in the laboratory. We consider these worms sufficiently similar to *P. longa* so as to be regarded as the same species. Although we have examined several thousand specimens of this species we have not observed a single specimen which could be confused with the holotype of *P. inglei*.

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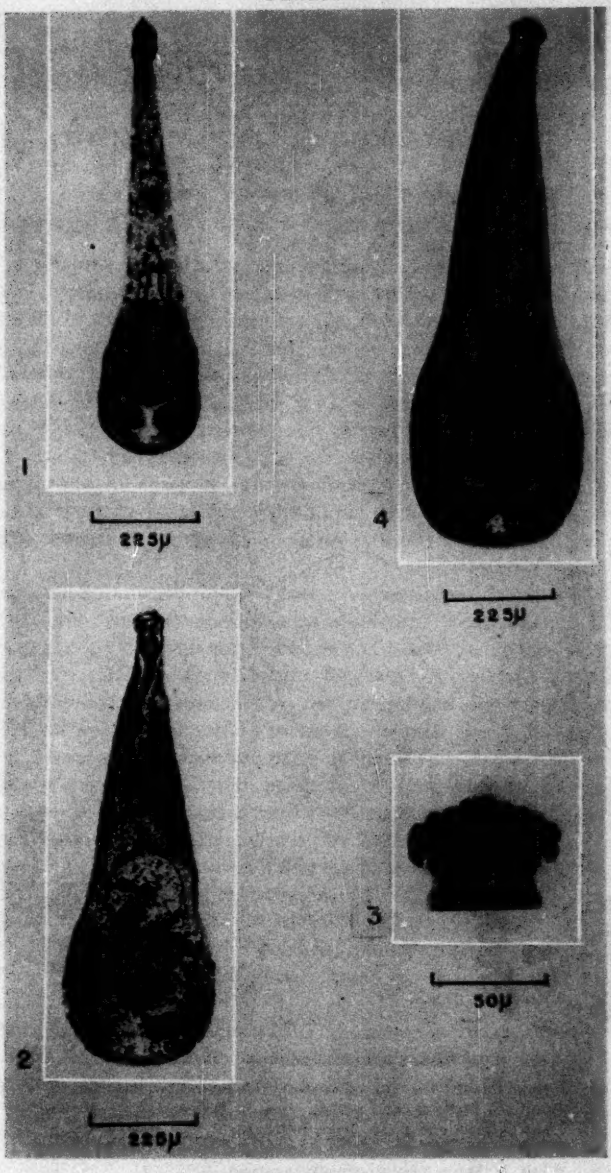
PLATE I

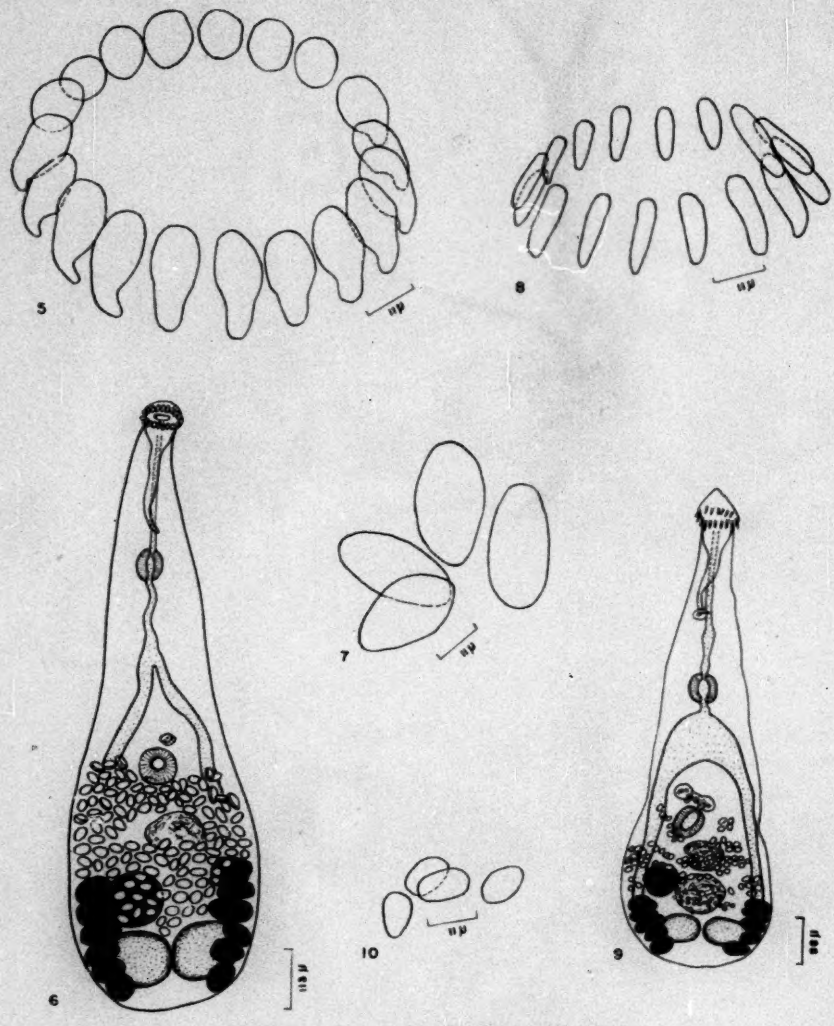


EXPLANATION OF PLATES

(All Photomicrographs)

FIGURE 1. Adult, *Phagicola longicollis* Kuntz and Chandler, 1956, ventral view.
FIGURE 2. Adult, *P. longa* (Ransom, 1920) Price, 1932, ventral view of syntype.
FIGURE 3. Mouth of *P. longicollis* surrounded by a coronet of seventeen spines, ventral view.
FIGURE 4. Adult, *P. inglei* n. sp., ventral view of holotype.

PLATE II



(All figures drawn with aid of camera lucida)

FIGURE 5. *P. inglei* n. sp., showing coronet of nineteen oral spines, ventral view of holotype.
FIGURE 6. Adult, *P. inglei* n. sp., ventral view of holotype.
FIGURE 7. Eggs of *P. inglei* n. sp. from holotype.
FIGURE 8. *P. longa* showing coronet of sixteen oral spines, ventral view of syntype.
FIGURE 9. Adult, *P. longa*, ventral view of syntype.
FIGURE 10. Eggs of *P. longa* from syntype.

THE MARINE, DERMATITIS-PRODUCING CERCARIA OF *AUSTROBILHARZIA VARIGLANDIS* IN CALIFORNIA (TREMATODA: SCHISTOSOMATIDAE)

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A search for schistosome cercariae from marine snails was begun in 1954 on the basis of an inquiry received from Dr. David Frost, who, at that time, was Health Officer of the City of Alameda, California. Dr. Frost was concerned about many reports related to a dermatitis of bathers. Certain areas in San Francisco Bay adjacent to Alameda were presumed to be sources of the dermatitis.

A marine snail, *Nassarius obsoletus* (Say), is the dominant gastropod in the suspected areas. The study of Stunkard and Hinchliffe (1952) showed that cercariae from this snail were responsible for schistosome dermatitis on the Atlantic Coast. During the present study, it was determined that the California *N. obsoletus* harbors a schistosome cercaria capable of producing dermatitis on human volunteers. We consider the cercaria to be the same species as that reported by Stunkard and Hinchliffe.

The species in question was first described as *Cercaria variglandis* by Miller and Northup (1926), who likewise found it in *N. obsoletus*. Stunkard and Hinchliffe experimentally produced the adult stage in birds and placed the species in the genus *Microbilharzia* Price. This genus was later suppressed by Penner (1953a) as a synonym of *Austrobilharzia* Johnston. *A. variglandis* and other members of the genus have been the subjects of studies pertaining to their life cycles and relation to dermatitis in several widely separated parts of the world. *Cercaria littorinalinae* was described by Penner (1950), who, using this species, was the first to demonstrate that a schistosome from a marine snail could produce dermatitis. The species was later placed in the genus *Austrobilharzia* by Penner (1953b). The cercariae of *A. littorinalinae* were reported from *Littorina planaxis* Philippi from Northern Mexico and Southern California. *A. variglandis* has been reported from another marine snail, *Littorina pintado* Wood, in Hawaii by Chu (1952) and Chu and Cutress (1954). The cercariae of *A. terrigalensis* Johnston occur in Australia, from *Pyrazus australis* Quoy and Gaimard of salt water lagoons, according to Bearup (1955, 1956).

On the Pacific Coast, the occurrence of *N. obsoletus* is interesting. This snail is thought to have been introduced from the Atlantic and, according to Demond (1952), it is known to occur on the Pacific Coast only in San Francisco Bay, California, and Boundary Bay, British Columbia. It is generally believed that the San Francisco introduction was a consequence of early attempts to establish eastern oysters in the bay.

In view of the fact that distinctions between schistosome species are often quite subtle, a rather detailed examination was made of our species, adults and cercariae.

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In addition to the identification of the schistosome, we attempted to gather some information on host-parasite relations pertaining to *N. obsoletus*. We were particularly interested in determining whether the presence of infection has a definite relation to snail size.

METHODS

Adult schistosomes were obtained from the mesenteric veins and liver of a female canary, whose legs were experimentally exposed to cercariae. Some of the flukes were examined in the living condition in 2.0% saline. All of them were eventually fixed in hot Gilson's fluid and stained with either borax carmine or alum carmine.

Both living and fixed cercariae were studied. The procedure for fixation employed by Stunkard and Hinchliffe (1952) was followed. Sea water, containing dispersed cercariae, and hot 10% formalin were mixed in equal parts. Only cercariae which had naturally emerged were fixed.

Snails were collected by hand at low tide. Size was determined as shell length (height) to the nearest millimeter. This dimension is somewhat inaccurate as an indicator of development of the larger snails, due to a varying degree of shell erosion which is noticeable in individuals approximately 17 mm or longer.

Observations on the prevalence of infection were made primarily from crushed snails. However, other snails were isolated in 4-ounce jars with lids. It was found that schistosome cercariae were not always observed during the first 24 hours of isolation. Other trematodes, represented by 4 additional species, emerged from many of the snails within the first 24-hour period. Schistosome infections could often be located by crushing or holding for further isolation the snails which seemed to be negative after 24 hours.

IDENTIFICATION OF THE SCHISTOSOME

The schistosome from Alameda was identified as *Austrobilharzia variglandis* (Miller and Northup, 1926) Penner, 1953, on the basis of its close agreement with the descriptions of the adult and the cercaria as given by Stunkard and Hinchliffe (1952).

Our cercariae exhibited some demonstrable differences in measurement when compared with the description by Stunkard and Hinchliffe (1952) of Rhode Island specimens from the same snail species. The measurements of Chu (1952), who was concerned with a different snail host, are somewhat more similar to those of Stunkard and Hinchliffe than to ours. The size of our specimens (as indicated by the lengths of the tail stem, furcae, and body) is greater than in either of the other 2 populations. When the mean values of these measurements are compared among our several lots of cercariae, each lot from a separate snail, the values show considerable variations. There were 7 snails from which 20, 46, 20, 45, 61, 20, and 20 cercariae were fixed and measured. The mean values in mm from these lots are respectively: body length, 0.250, 0.275, 0.241, 0.258, 0.255, 0.246, 0.263; tail stem length, 0.264, 0.315, 0.285, 0.304, 0.264, 0.280, 0.278; furcal length, 0.161, 0.216, 0.164, 0.206, 0.180, 0.179, 0.196. In comparison, the means given by Stunkard and Hinchliffe are: body length, 0.237; tail stem length, 0.228; and furcal length, 0.126. With respect to furcal length, in which variability is pronounced, our mean values are from 0.035 to 0.090 longer than the mean given by Stunkard and Hinchliffe. Among our 7 lots, mean body lengths are only slightly variable. The corresponding tail stems are slightly to considerably longer than the bodies. This is not the case with the Rhode Island material, in which body length is slightly greater than tail stem length.

Of considerable importance in the differentiation among schistosome cercariae is the nature of the granulation of the penetration glands. Meyer and Dubois (1954) divided the schistosomes with pigmented eyespots into 2 groups, those species in

which the anterior glands (exclusive of the escape glands) are more coarsely granular than the posterior ones (Group I) and those species in which the posterior glands are more coarsely granular (Group II). In agreement with Stunkard and Hinchliffe we find that the cercaria of *A. variglandis* has the posterior glands more coarsely granular than the anterior glands and should be referred to Group II in the Meyer and Dubois' classification. Meyer and Dubois have placed the species, with a question mark, in Group I. The original description of the cercaria, by Miller and Northup (1926), leaves some doubt as to the difference in granulation between the two sets of glands. These authors remark that the anterior ones have "coarsely granular" cytoplasm and that the posterior ones have "rod-filled cytoplasm, homogenous in sections." If this description could be interpreted to mean that the anterior glands contain coarse granules and the posterior glands have coarser granules, then the description is compatible with our observations on living specimens. When the cercaria has been fixed in formalin, both groups of glands lose their granular appearance.

The position assumed by resting cercariae has been used by recent workers as a method of separating some of the schistosomes, particularly certain species of fresh water origin. Stunkard and Hinchliffe (1952) have observed that the cercaria of *A. variglandis* comes to rest on the water surface and assumes a position in which the tail is curved, coming to lie beside the body. The same sort of position was observed in *A. terrigalensis* by Bearup (1955, 1956). In addition to the curved condition, there are other features of the manner of resting which should be mentioned. *A. variglandis* was noted in the present study to make contact with the surface film along the entire tail and the posterior end of the body. The anterior portion of the body is bent slightly under the surface. It is the dorsum of the cercaria which is uppermost, so that the acetabulum does not contact the surface film. Other species which rest at the surface are described as clinging by means of the acetabulum (in those species where information on resting position is reported in detail).

It was also observed that the cercariae are positively phototactic, but this is only noticeable during a rather brief period of motility following emergence. The result is that they tend to congregate at the light side of the container before assuming the resting position at the surface. After this position is once assumed, the cercariae may swim, but usually for short distances only.

PREVALENCE OF SNAIL INFECTIONS

Nassarius obsoletus is extremely abundant along the shore of San Francisco Bay in the vicinity of Alameda. The area is primarily mud flat, and many of the snails are exposed at low tide. On June 23, 1955, it was estimated that there were approximately 170 snails per square foot along a 100-yard segment of the shoreline adjacent to one of the Alameda bathing areas. There was no obvious seasonal change in abundance, but the snails were more frequently found to be buried under the surface of the mud during the winter than during the rest of the year.

During 1955 and 1956 snails were collected and examined from an area covering 1300 yards along the southern shore of Alameda. In Table I the 3852 snails taken in 11 collections from May, 1955 through April, 1956 and examined by crushing, are arranged in 5-mm size classes. Large specimens make up a somewhat greater proportion of the collections than would be expected if strictly random samples had been taken. Thirty snails were infected with *A. variglandis*. None of

these infections occurred in the 1724 individuals measuring less than 20 mm. The infected snails ranged in size from 21 to 35 mm.

Observations were made on the 4 species of trematodes, other than the schistosome, infecting *N. obsoletus*. The prevalence of total trematode infection was observed in the same group of snails, and the same kind of minimal size-infection relation was noted (see Table I). The smallest individual observed to be infected

TABLE I. *Prevalence of infection in crushed snails by size class.*

Date of Collection	5–9 mm			10–14 mm			15–19 mm			20–24 mm			25–29 mm			over 29 mm			all sizes		
	N	T	S*	N	T	S	N	T	S	N	T	S	N	T	S	N	T	S	N	T	S
VI–23–55	10	0	0	34	0	0	54	6	0	68	35	0	25	25	0	2	2	0	193	68	0
VII–14–55				11	0	0	114	3	0	66	19	0	15	15	0				206	37	0
VIII–5–55				17	0	0	42	0	0	21	2	0							80	2	0
VIII–30–55				2	0	0	62	0	0	108	27	1	39	34	0	1	1	0	212	62	1
IX–16–55	22	0	0	54	0	0	135	4	0	163	74	0	30	27	0	2	2	0	406	107	0
X–7–55	6	0	0	41	0	0	166	3	0	228	139	4	68	66	1	8	8	1	517	216	6
XI–2–55				15	0	0	169	7	0	350	152	2	113	108	3	10	10	0	657	277	5
XI–29–55	4	0	0	37	0	0	153	6	0	332	153	7	116	112	5	5	5	0	647	276	12
I–3–56	6	0	0	41	0	0	167	9	0	145	53	1	14	14	2				373	76	3
III–21–56	9	0	0	39	0	0	160	0	0	108	25	1	9	8	0				325	33	1
IV–16–56	14	0	0	20	0	0	120	3	0	72	15	0	10	10	2				236	28	2
Totals	71	0	0	311	0	0	1342	41	0	1661	694	16	439	419	13	28	28	1	3852	1182	30

* Prevalence of infection is expressed as: Number of snails examined (N), number infected with any trematode (T), and number infected with *A. variglandis* (S). The numbers examined on each date do not represent random samples with respect to size.

with any trematode was 17 mm. Infections are rare in the category of snails 15 through 19 mm, and almost all of those 25 mm or larger were infected. Within the 20- through 24-mm group, in which the transition seems to occur, there could have been only slight seasonal fluctuation in the size-infection relation. The minimal size to be infected at a rate not less than 50% varied between 22 mm and 24 mm from month to month.

Not included in Table I is a group of 1217 large snails examined by isolation. Consequently, 23 additional *A. variglandis* infections were observed, in hosts from 24 to 30 mm in length. Data on the occurrence of infections in both crushed and isolated snails cover the period from May, 1955 through July, 1956, with at least 1 collection in each month except December. From the available data, schistosome-infected snails were recorded for every month except May and September. The only evidence of new infections was the occurrence of what were believed to be migrating daughter sporocysts in three individuals taken on January 3, 1956.

DISCUSSION

Stunkard and Hinchliffe (1952) mentioned that schistosome infections are of highest incidence in the larger *Nassarius obsoletus*. The present data indicate that this is true in the Alameda snails and that small individuals of a considerable size range are free from observable trematode infections of any kind. It is believed that even the smallest snails in the sample are old enough to have been exposed to miracidia and to have developed noticeable infections were it not for some preventive mechanism. Further study is necessary to determine the nature of this mechanism.

From the data collected for more than a year in snails crushed or isolated, it is evident that *A. variglandis* infections are apt to exist throughout the year in this climate. Rates of schistosome infection for individual collections are so low that it is difficult to tell if fluctuations occurred. Although figures for actual size distributions are not available, it was estimated that snails less than 20 mm were in the

majority throughout the study period. The remaining snails tended to show a small infection rate which probably reaches a maximum at a size well over 20 mm. Sindermann (1956) reports *A. variglandis* infection rates in northern New England *N. obsoletus* as high as 31% in the autumn in the high tide zone. In our California situation the greatest rate of infection occurred in the November 29 collection. Considering only those snails which were 20 mm or over, the rate was 2.6% in 453 snails. According to Sindermann, the high infection rates were observed among snails which failed to make an offshore migration in the autumn as is normal for snails in that area. No noticeable migratory tendency was observed during the period of study in California. Possibly, a more uniform temperature is responsible for a more stable population.

SUMMARY

The cercaria of *Austrobilharzia variglandis* (Miller and Northup, 1926) Penner, 1953, is reported from Alameda, California, in San Francisco Bay. The snail host is *Nassarius obsoletus* (Say). The cercaria is implicated as a cause of marine schistosome dermatitis among bathers in Alameda.

The California cercaria, though larger in certain dimensions than those described from Rhode Island and Hawaii, is the same species. Measurement variations exist between different lots of cercariae (each lot from a separate snail). Additional notes are made as to the morphology and behavior of the cercaria.

Studies on the relationship between snail size and prevalence of infection reveal that *A. variglandis* infections are likely to be found only in snails over approximately 20 mm in length in this population. The same kind of size relation applies to total trematode infection, concerning 5 species parasitizing *N. obsoletus*. *A. variglandis* infections were detected in small numbers through the year.

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Dr. Leo G. Hertlein and Mr. Allyn G. Smith of the California Academy of Sciences kindly identified the snails.

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RESEARCH NOTE

ON THE PATHWAY OF CARBOHYDRATE METABOLISM IN *ENTAMOEBA HISTOLYTICA*

In a thorough and excellent series of enzyme studies on the intermediary carbohydrate metabolism of *E. histolytica*, Hilker and White (1958, Fed. Proc. **17**: 242; 1958, J. Biol. Chem., in press) have recently shown that this ameba, in addition to possessing enzymes of the glycolytic scheme for using glucose, also contains enzymes of an alternate pathway. The latter pathway was first discovered in the bacillus *Pseudomonas saccharophila* (Entner and Doudoroff, 1952, J. Biol. Chem. **196**: 853–862), and since has been found to occur predominantly in a variety of polar-flagellated bacteria. The report of Hilker and White is the first to indicate this pathway to be present in protozoa or any animal cell. It was, therefore, thought that it would be of interest to check their findings with the use of radioactive glucose labelled in the 1-carbon atom.

Lactic acid has previously been shown to be one of the fermentation products of *E. histolytica* (Entner & Anderson, 1954, Exp. Parasitol. **3**: 234–239). In the glycolytic pathway, lactic acid from glucose-1-C^{14} should be labelled in the methyl group, whereas in the *P. saccharophila* pathway, the labelling should appear in the carboxyl group. Alternatively, in the pentose-phosphate pathway, the labelling from glucose-1-C^{14} would be chiefly in respiratory CO_2.

Our results (Table I) confirm the findings of Hilker and White, that *E. histolytica* does indeed possess the pathway found previously only in bacteria. The lactic acid from glucose-1-C^{14} is labelled predominantly in the carboxyl group. The considerable labelling in the methyl group and lack of labelling of CO_2 also confirms the report of Hilker and White of the presence of glycolysis and absence of the pentose-phosphate pathway.

TABLE I

Lactic acid formed	5.76 micromoles
Radioactivity (counts/min.)	
Respiratory CO_2	0
Lactic acid	450
— COOH	218
— CHOH	47
— CH_3	185

In the experiment, the SH strain of *E. histolytica* was used and was grown on the Shaffer-Frye medium. The amebae were washed free of bacteria, and 2.9×10^6 amebae were supplied with glucose-1-C^{14} and incubated in an atmosphere of nitrogen for 1 hour at 37° C. The fermentation acids were separated from the incubation medium by ether extraction, and lactic acid was isolated on a column of silica gel. The method used for degradation of lactate was that described by Abraham and Hassid (1957, Meth. in Enzymology, **4**: 530–535).

Although microscopic examination showed the washed amebae to be reasonably free of bacteria, it was deemed important to investigate the existence of the alternate pathway in the streptobacillus. Accordingly, experiments were conducted both with enzyme preparations, made by alumina grinding and in which phosphogluconic acid was the substrate, and with whole cells supplied with glucose-1-C^{14}. From 10 to 15% of the fermentation products of the streptobacillus is in the form of lactic acid, the bulk being steam-volatile acids. Both the enzyme experiments and the insignificant amount of labelling in the carboxyl group of lactate produced from the radioactive glucose, indicated that the *P. saccharophila* pathway is absent in the streptobacillus.

This investigation was supported by a research grant [# E-1861(C-1)] from the National Institute of Allergy and Infectious Diseases, NIH, USPHS. The author would like to acknowledge the assistance of Lemuel Evans and Celia Gonzalez.—NATHAN ENTNER, *Dept. of Preventive Medicine, New York University College of Medicine, New York 16, N. Y.*

PRIMATOTREMA MACACAE GEN. NOV., SP. NOV. FROM MACAQUE RHESUS MONKEYS, AND A REDESCRIPTION OF *PHANEROPSOLUS OVIFORME* POIRIER (1886) LOOSS, 1899 (LECITHODENDRIIDAE)*

PREMVATI**

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An examination of the intestines of more than 1,000 Macaque Rhesus monkeys for all helminths showed only 4 to be infected with the trematode described below, the number in each case being 18, 12, 10 and 6, respectively.

The trematodes were fixed in AFA, Bouin's fluid and Coatney's fixative, preserved in 70% alcohol, and mounted in Borax carmine, Aceto-alum carmine, Gower's alum-carmine, and Ehrlich's hematoxylin. The measurements are based on 10 specimens, and are in millimeters.

Primatotrema macacae gen. nov., sp. nov.

Description. A small, spined oval trematode, broadest in the middle, narrow at the ends, the anterior end being narrower than the posterior (Fig. 1). Length 0.72 to 0.78, width 0.40 to 0.49. Oral and ventral suckers of almost equal size, 0.108 to 0.114 in diameter. Ventral sucker just above equator line. Mouth subterminal; pharynx well developed, broader than long, and measuring 0.04 by 0.057; esophagus absent; intestinal ceca sac like, very short, terminating mid-way between pharynx and ventral sucker. Intestinal ceca barely visible in mounted specimens. Excretory bladder V-shaped, opening at posterior-terminal end of body, anterior limbs of 'V' long, extending on each side to mid-body.

Testes 2, slightly ovoid, 0.153 by 0.131, situated one on either side of the body in front of ventral sucker in region of cirrus pouch and behind pharynx. Cirrus pouch very long, 'S'-shaped, in front of ventral sucker, containing an oval vesicula seminalis interna; elongated pars prostatica slightly broader at its base, leading to small ductus ejaculatorius which ends in a long penis.

Ovary somewhat oval, 0.141 by 0.108, situated on right of ventral sucker completely posterior to right testis. Receptaculum seminalis well developed, 0.091 in diameter, posterior to ovary and ventral sucker. Ootype just posterior to mid-body. Laurer's canal present. Vitellaria consist of 6 to 8 follicles on either side of body, anterior and slightly dorsal to anterior portion of testes. Vitellaria open on each side into vitelline duct. Right vitelline duct longer than left, crossing testis, cirrus pouch and ventral sucker to meet left vitelline duct which crosses left testis posterior to ventral sucker in mid-body, to open into ootype by a common aperture. Uterus highly convoluted filling whole body posterior to vitelline follicles, having both descending and ascending limbs (Fig. 2). Metraterm runs slightly towards left of ventral sucker, ventral to cirrus pouch, opening by genital pore in vicinity of oral sucker.

Eggs (Fig. 3) 0.028 to 0.031 in length, 0.0116 to 0.0156 in width. Eggs fully mature inside uterus, showing fully developed miracidia.

Genital pore median, anterior to pharynx, opening in vicinity of oral sucker and clearly seen in lateral view (Fig. 4). This feature is characteristic in all specimens.

Host: Macaque rhesus monkey.

Location: Small intestine.

DISCUSSION

Primatotrema most closely resembles *Phaneropsolus* (Looss, 1899) of which the following species have been described from primates (Nicoll, 1927): *P. longipenis*

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** Margaret McWilliam Fellow (awarded by Canadian Federation of University Women). At present on leave from the Department of Zoology, University of Lucknow, India.

(Looss, 1899) from apes in Egypt, *P. oviforme* (Poirier, 1886) Looss, 1899 from *Nycticebus javenicus* Geoff, *P. orbicularis* (Diesing, 1850) Braun, 1901 from *Nyctipithecus trivirgatus* Humboldt, and *P. lakdivensis* Fernando, 1933 from *Loris tardigradus* from Ceylon.

In these species, as well as in those described from birds, the genital pore is situated immediately behind the pharynx whereas in all specimens of *P. macacae* examined, it is well anterior to the pharynx in the vicinity of the oral sucker. *P. macacae* also differs from *Phaneropsolus* in that the ovary is towards the right of the ventral sucker in the former and towards the left in the latter; in having a well developed and larger receptaculum seminalis; in the absence of the esophagus, and in having very small intestinal ceca. The position of the ovary and particularly the genital pore and the absence of the esophagus are the criteria used in creating the new genus.

There has been some controversy concerning the position of the genus *Phaneropsolus* in the family Lecithodendriidae. Mehra (1935) formed 6 sub-families of Lecithodendriidae and included this genus in a new sub-family—Phaneropsolinae. Macy (1936), Lal (1939), Kaw (1950) and Neiland (1951) included it in the sub-family Pleurogenetinae Looss, 1899. Srivastava (1934), Dawes (1946) and Skarbilovich (1943) have placed the genus *Phaneropsolus* under the sub-family Lecithodendriinae. Skarbilovich created a new tribe 'Phaneropsolea' under the sub-family Lecithodendriinae, the diagnostic characters being based on the genital opening situated median with exception of the genus *Mosesia*, but shifted considerably anterior from the ventral sucker, being nearly always situated near the pharynx, with cirrus pouch well developed. He has included under this tribe *Phaneropsolus* Looss, 1899, *Eumegacetes* Looss 1900, *Loxogenes* Stafford 1905, *Mosesia* Travassos 1928, *Pleuropsolus* Mehra 1935, *Exotidendrium* Mehra 1935, *Anentrotrema* Stunkard 1938, and *Chiroptodendrium* Skarbilovich 1943.

It seems probable that Kaw (1950, 1953) and Neiland (1951) have overlooked the classification of the whole family given by Skarbilovich (1943). Kaw agrees with Lal (1939) in dividing the family into 2 sub-families, Lecithodendriinae and Pleurogenetinae, on the basis of the character, 'presence and absence of cirrus sac.' Neiland (1951) is in agreement with Macy (1936, 1940) and Gogate (1939) in creating a new sub-family 'Gyrobascinae' to include the 5 genera *Ganeo* Klein 1905, *Gyrabascus* Macy 1935, *Opiosaculus* Macy 1935, and *Myotitrema* Macy 1940. He considered the sub-family 'Palitreminae' Gogate as a synonym of Pluerogenetinae.

The author is in agreement with the classification given by Skarbilovich (1943). He retained only 2 sub-families Lecithodendriinae and Pleurogenetinae, which are further divided into tribes to include the various genera. *P. macacae* may be included under the tribe Phaneropsolea Skarbilovich 1943, and the sub-family Lecithodendriinae. A slightly amended key for the genera of the tribe Phaneropsolea Skarbilovich 1943 is given as follows:

Key to the genera of the tribe Phaneropsolea Skarbilovich 1943.

1. Genital pore shifted to left and situated mid-way between pharynx and ventral sucker *Mosesia* Travassos 1928.

2. Genital pore median, anterior to ventral sucker.

A. Vitellaria in clusters of follicles in anterior part of body.

(a). Cirrus pouch elongate.

(i). Genital pore immediately behind the pharynx ..... *Phaneropsolus* Looss 1899.

(ii). Genital pore far anterior to pharynx ..... *Primatotrema* gen. nov.

(iii). Genital pore mid-way between two suckers.

(a'). Cirrus pouch does not reach ventral sucker ..... *Loxogenes* Stafford 1905.
(b'). Cirrus pouch overlaps ventral sucker and partially situated anterior to ventral sucker ..... *Pleuropsolus* Mehra 1935.
(b). Cirrus pouch spherical ..... *Chiroptodendrium* Skarbilovich 1943.

B. Vitellaria in posterior part of body.
(a). Cirrus pouch short and does not go beyond ventral sucker.
(i). Intestinal branches long, testes anterior to ventral sucker *Eumegacetes* Looss 1900.
(ii). Intestinal branches absent, testes behind ventral sucker *Anentrotrema* Stunkard 1938.
(b). Cirrus pouch long, going behind ventral sucker, seminal vesicle straight *Exotidendrium* Mehra 1935.

On two occasions *Phaneropsolus oviforme* were obtained from the small intestines of rhesus monkeys. Altogether 25 specimens were collected. The description given by Poirier and Looss for *P. oviforme* varies to a certain extent in the sizes of different structures and also in the total body size. It was therefore considered worthwhile to redescribe the form.

Phaneropsolus oviforme (Poirier, 1886) Looss, 1899
(Redescription—measurements in millimeters)

Body is small, measuring 0.97 to 1.092 with a width of 0.51 to 0.59 (previous measurement being 0.66 by 0.45). Cuticle spiny, suckers equal with a diameter of 0.119 (previous being 0.16); ventral sucker situated anterior to middle of the body; alimentary canal with pharynx, esophagus and intestinal ceca; excretory bladder 'V' shaped, ending near ventral sucker. Testes rounded anterior to ventral sucker, measuring 0.131 to 0.159 in diameter; ovary 0.131 in diameter; receptaculum seminalis behind the ovary, 0.074 in diameter, practically half of the ovary; Laurer's canal present. Vitelline follicles 7 to 9 on either side of the body, anterior to testes and intestinal ceca; vitelline ducts join behind the ventral sucker; uterus filling the entire midbody. Eggs very numerous measuring 0.031 by 0.014 as against the previous description 'eggs not very numerous and measure 0.026 by 0.015.'

ACKNOWLEDGMENT

The author is grateful for the kind cooperation of the Laboratory of Hygiene, Department of National Health and Welfare, and the Institute de Microbiologie, Université de Montréal in supplying the viscera of rhesus monkeys. She also wishes to thank the Institute of Parasitology, Macdonald College of McGill University, P. Q., Canada, for providing all necessary facilities.

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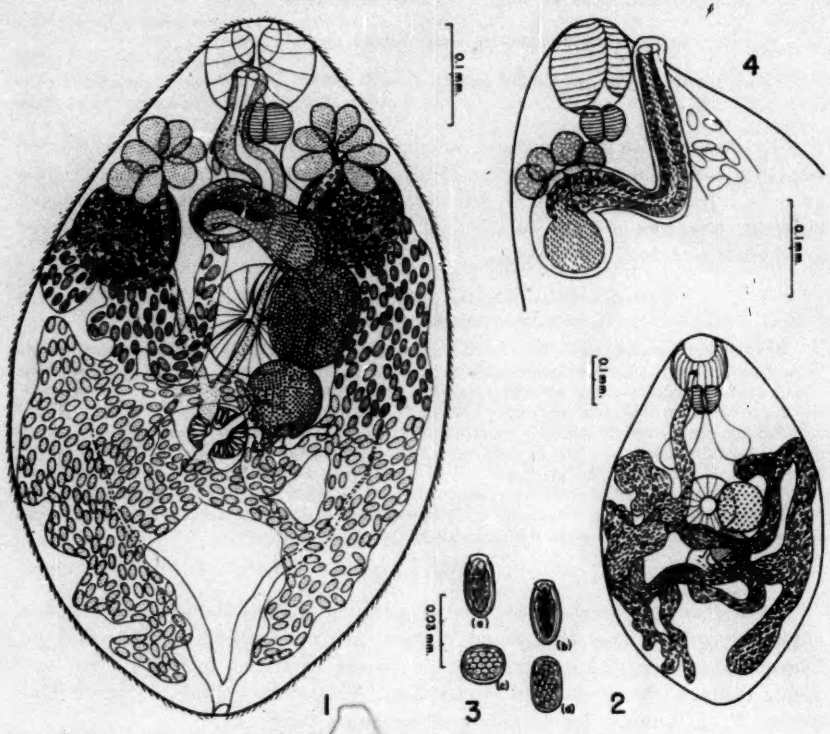

EXPLANATION OF PLATE

FIGURE 1. *Primatotrema macacae* gen. nov., sp. nov. Dorsal view of the adult.

FIGURE 2. *P. macacae.* Disposition of the uterus.

FIGURE 3. *P. macacae*—(a) and (b). Mature eggs with miracidia; (c) and (d). Early stages of eggs.

FIGURE 4. *P. macacae.* Anterior end of the lateral view to show the position of the genital pore.

MAZOCRAEOIDES OLENTANGIENSIS, N. SP., A MONOGENETIC TREMATODE PARASITIC ON THE GILLS OF THE GIZZARD SHAD, *DOROSOMA CEPEDIANUM* (LE SUEUR)

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The genus *Mazocraeoides* Price, 1936 contains *M. georgei* Price, 1936, *M. dorosomatis* (Yamaguti, 1938) Sproston, 1946, *M. prashadi* Chauhan 1950, and *M. opisthonema* Hargis 1955. All these worms are parasitic on clupeid fishes. The purpose of this paper is to describe a fifth species which parasitizes a fresh-water clupeid, the gizzard shad. A succeeding paper will describe the development of this trematode.

Collections of the host were made each month from July through November, 1957, from the Olentangy River, Franklin County, Ohio. The gills of 91 gizzard shad, *Dorosoma cepedianum* (Le Sueur), were examined; 68 of the fish or 74.7% were infected; the infections varied in number from 1 to 24. The worms were relaxed by refrigeration and fixed with Lavdovsky's fixing reagent (AFA). Both sections and whole mounts which were stained with either Harris' haematoxylin or Semichon's stain were studied. All drawings were made with the aid of the camera lucida. All measurements are in millimeters; the numbers in parentheses represent the extremes of 10 measurements, while the number preceding the parenthesis indicates the mean of these figures. The terminology used is largely that of Hargis (1954).

Mazocraeoides olentangiensis n. sp.

Order Monogenea Carus, 1936
Suborder Polyopisthocotylea Odhner, 1912
Superfamily Diclidophoroidea Price, 1936

Diagnosis: Family Mazocraeidae. Body form clavate, 1.2 (1.0–1.8) long by 0.4 (0.3–0.6) (maximum width) with anterior end narrow and posterior end greatly broadened. Prohaptor a pair of buccal suckers. Opisthaptor 4 pairs of clamps located on ventro-lateral surface of posterior half of body. Anterior pair of clamps 0.047 (0.040–0.056) long by 0.058 (0.050–0.069) wide; second pair of clamps 0.050 (0.041–0.058) long by 0.051 (0.049–0.075) wide; third pair, 0.048 (0.042–0.061) by 0.059 (0.054–0.072) wide; fourth pair, 0.048 (0.042–0.058) by 0.054 (0.049–0.064) wide. Clamps of typical mazocraeid type. Anterior loop U-shaped, with posterior ends bent mesially, apparently articulating with, but not confluent with, posterior loop element. Posterior loop incomplete, consisting of a heavily cuticularized portion which articulates between ends of the anterior loop, and a lightly cuticularized, more flexible, flap-like anterior portion. Middle loop complete, U-shaped, with a medial scoop-shaped portion. Centerpiece fenestrated. Two pairs of anchors located on a terminal lappet. Lateral pair large with broad shaft, guard and curved blade, 0.063 (0.058–0.067) long; inner pair smaller, slightly sigmoid-shaped, 0.027 (0.022–0.027) long. Between large and small anchors on each side is a flask-shaped, hooklet tip-guard, 0.008 (0.007–0.010) long. Mouth sub-terminal. Pharynx elongate ovoid, 0.057 (0.050–0.062) long by 0.034 (0.031–0.036) wide, located immediately posterior to buccal suckers. Esophagus unbranched anterior to genital corona, branched posterior to latter structure and bifurcating into 2 intestinal crura approximately ¼ length of worm from anterior tip of body. Crura highly ramified, not confluent posteriorly. Testis saccate, largely to left (or right) of mid-line, may occupy entire space between ovary and left (or right) or intestinal crus. Proximal portion of vas deferens narrow, difficult to discern, expanding into a seminal vesicle anterior to vitel-

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line reservoir and narrowing anteriorly as it passes in mid-line dorsal to uterus. Common genital pore in mid-ventral line. Genital corona consisting of an ovoid muscular piece, 0.027 (0.026–0.030) long by 0.022 (0.020–0.029), armed along the opening of male duct by 2 rows of 5 longitudinally arranged, S-shaped spines and laterally by a pair of larger bi-partite hooklets, 0.015 (0.014–0.016) long. Attached to lateral margins of genital corona on either side is a band of diagonally arranged muscle fibers. Ovary elongate, folded, to right (or left) of mid-line, consisting of descending and ascending limbs. Oviduct short, joined by branch of genito-intestinal canal, passing dorsally and emptying into ootype. Ootype dorsal to vitelline reservoir, sursounded by Mehlis' gland. Uterus in mid-line, ventral to vas deferens, consisting of a proximal muscular portion, a middle ciliated portion and a short terminal segment devoid of cilia; terminal portion may be greatly dilated. Vitelline ducts uniting and forming a ventrally located Y-shaped vitelline reservoir which passes posteriorly, then curves anteriorly, passing dorsal to oviduct and joining ootype. Left (or right) vitelline duct receives an accessory duct extending from a dorsal, median, ciliated canal located just anterior to gut bifurcation; accessory duct passes in mid-line dorsal to vas deferens, then veers laterally partially concealed by vitellaria. Genito-intestinal canal extending from right (or left) intestinal crus and joined by separate branches to both oviduct and vitelline reservoir. Vitellaria follicular, located dorsal, ventral, and lateral to gut, and extending from level of genital pore to near posterior end of body. Vaginal pore not observed. Egg elongate ovoid, 0.177 (0.159–0.211) long by 0.059 (0.050–0.070) wide, operculum suture approximately 0.020 from anterior end of egg.

Type specimens: Holotype, U. S. National Museum Helminth. Coll. No. 38339; paratypes, No. 38340.

DISCUSSION

It must be pointed out that Kimpel (1938) in a paper read before the American Society of Parasitologists, reported a monogenetic trematode, *Mazocraes cepedianum*, from the gills of gizzard shad collected from Lake Decatur, Illinois. *Mazocraes cepedianum* has subsequently been designated a *nomen nudum* by Sproston (1946). It is very possible that the worm presently described is the same species that Kimpel reported.

As pointed out by Hargis (1955), the genus *Mazocraeoides* is easily recognized by its club-shaped form; this contrasts sharply with the lanceolate body of the genera *Mazocraes*, *Kuhnia*, and *Clupeocotyle* of the family Mazocraeidae. Of the previously described species in this genus, *Mazocraeoides olentangiensis* most closely resembles *Mazocraeoides georgei*. It differs from the latter species in the following respects: (1) measurement of hard parts; (2) extent of the ovary; (3) morphology of the genital corona; (4) number of polar filaments of egg; (5) difference in hosts.

An apparently common feature of this species is the phenomenon of *situs inversus*. In 14 of 27 worms which were examined the sex organs are disposed as in Figure 1; in the other 13 the ovary is to the left of the mid-line, the testis on the right side and the genito-intestinal canal empties into the left intestinal crus. This same phenomenon was noted by Sproston (1945) in *Kuhnia scombri*.

The genus *Mazocraeoides* is said to have 3 pairs of anchors (Sproston, 1946); *M. olentangiensis* has 2 pairs plus a larval hooklet tip-guard between the 2 types of anchors. I have never observed the retention of the larval hooklets associated with the tip-guards in adult worms of this species, but have seen them in late larvae and earlier stages. The hooklets are sometimes seen in the adult stage of *Mazocraeoides georgei* or in some specimens may be missing (Slide No. 35623, USNM Helminthological Collection). In the case of *Mazocraeoides opisthonema*, Hargis (1955) figures and describes intermediate "anchors" which resemble the flask-shaped hooklet tip-guards of *M. olentangiensis* while Yamaguti (1938) figures small flask-shaped "hooks" between the 2 anchor types. In view of the fact that the tip-guards could hardly be called anchors and since their accompanying hooklets are not always present, it would seem preferable to describe the genus *Mazocraeoides* as

having only 2 pairs of anchors plus a pair of intermediate larval hooklets and/or their tip-guards. The nature of the larval hooklets will be discussed in a future paper which will describe the development of *Mazocraeoides olentangiensis*.

SUMMARY

Mazocraeoides olentangiensis n. sp., a monogenetic trematode parasitic on the gills of the gizzard shad, *Dorosoma cepedianum* (Le Sueur) is described. This brings to five the number of described species in this genus.

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EXPLANATION OF PLATE I (p. 646)

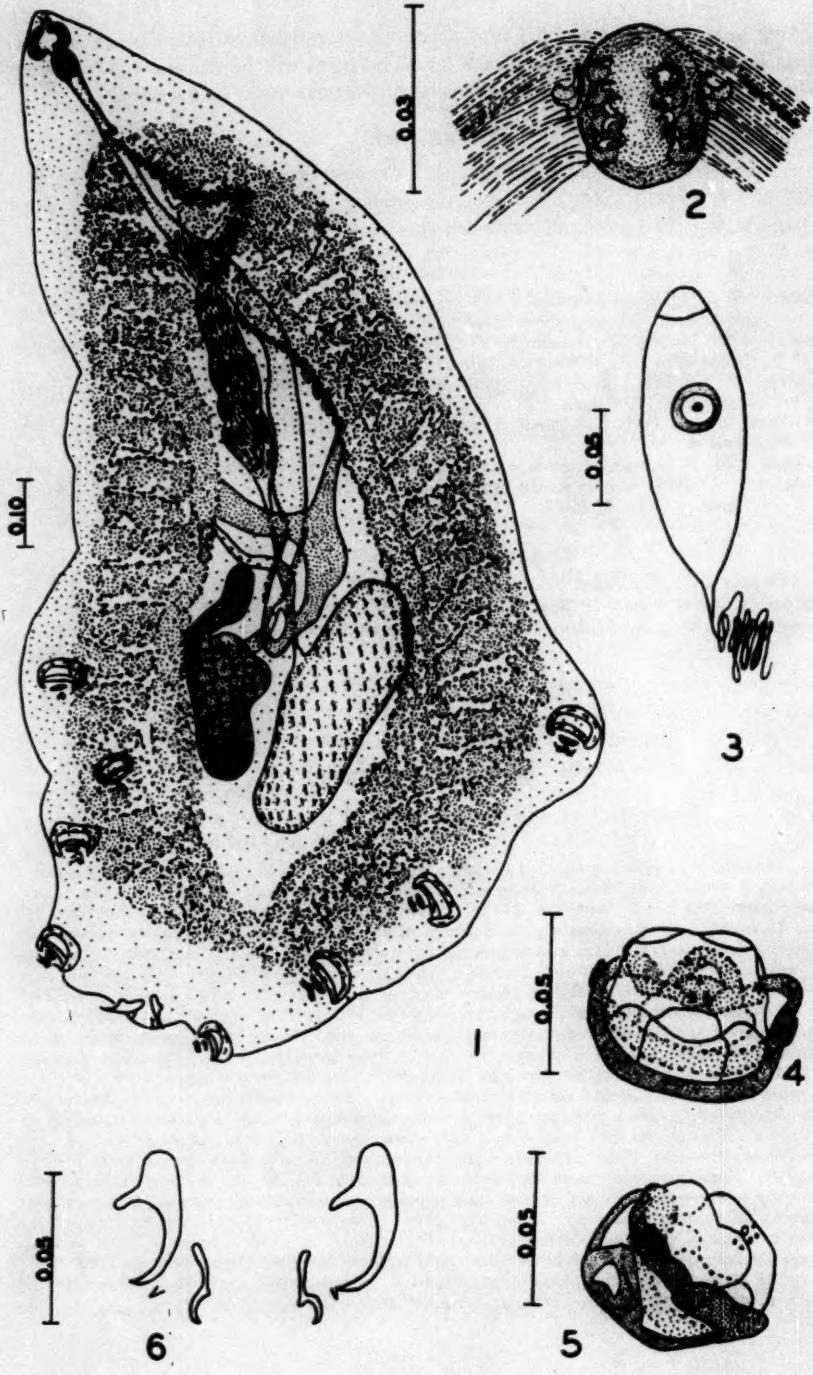
FIGURES 1–6. *Mazocraeoides olentangiensis* n. sp. 1. Whole worm, ventral view; 2. Genital corona, ventral view; 3. Egg; 4. Clamp closed, ventral view; 5. Clamp partially closed, dorso-lateral view; 6. Anchors and larval hooklet tip-guards.

RESEARCH NOTE

COMPLEMENT FIXATION TEST IN KALA-AZAR USING *MYCOBACTERIUM BUTYRICUM* ANTIGEN

Antigens from acid-fast bacilli (*M. tuberculosis, M. avium, M. phlei,* Kedrowsky bacillus) are widely used in complement fixation test for the diagnosis of kala-azar (Greval, Sen Gupta, and Napier, 1939, Ind. J. Med. Res. **27**: 181; Dharmendra and Bose, 1941, Ind. J. Med. Res. **29**: 7). *M. butyricum* is a non-pathogenic organism easily secured in large amounts by culturing in glycerine-broth medium. It is also commercially available in dry powder form (killed and dried *M. butyricum*, Difco Laboratories, Detroit 1, Mich.). Complement fixation tests for kala azar were tried, employing *M. butyricum* antigen prepared as follows: Ten ml of acetone were added to 100 mg of dried bacilli and extracted overnight at 37° C. The acetone was decanted after centrifugation and the remainder evaporated under vacuum. To the sediment 15 ml of methyl alcohol were added and the suspension was left at room temperature for 3 days with frequent shaking. The clear supernatant was used as antigen. This antigen was quite stable when kept at room temperature and was not anti-complementary. The optimal dilution for the test proper was determined by block titration. Complement fixation tests were performed, according to Kolmer's technique, on sera from 6 kala-azar patients and on 5 dogs naturally infected with *Leishmania donovani*. Positive results were obtained with all sera, titers ranging from 1:80 to 1:5,120. Negative results were obtained with sera from healthy persons and from patients suffering with chronic Chagas' disease, muco-cutaneous leishmaniasis, syphilis, schistosomiasis mansoni and tuberculoid leprosy. Cross reactions were observed in 40% of lepromatous lepers, in 3 out of 15 cases of active tuberculosis (low titers) and in one case of acute Chagas' disease. These results are similar to those obtained with tubercle bacillus antigen (Nussenzweig, 1957, Hospital, Rio de Janeiro **51**: 217).—J. PELLÉGRINO, Z. BRENER AND ULYSSES M. SANTOS, *Instituto Nacional de Endemias Rurais, Centro de Pesquisas de Belo Horizonte, Brasil.*

PLATE I



A NEW SPECIES OF *DICROGASTER* (TREMATODA, HAPLOPORIDAE) FROM *MUGIL CEPHALUS* IN THE GULF OF MEXICO

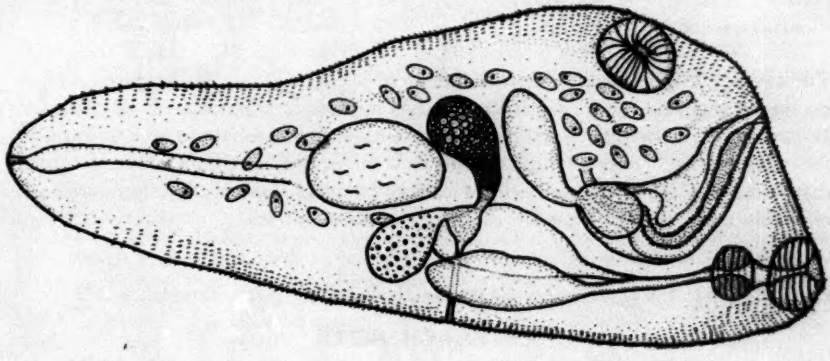
VERNON E. THATCHER* AND ALBERT K. SPARKS**

During recent studies of marine fish trematodes conducted at the Texas A & M Research Foundation laboratory at Grand Isle, Louisiana, numerous small Digenea were recovered from the mullet, *Mugil cephalus*. Some of these were found to belong to the family Haploporidae Nicoll, 1914. The genera in this family are known from European waters and from South America. Apparently, no representative of this family has yet been recorded for North America or the Gulf of Mexico, although members of the closely related family Waretrematidae occur here. Present specimens are believed to represent an undescribed species of *Dicrogaster* Looss, 1902, and the name *Dicrogaster fastigatus* is hereby proposed. The following diagnosis is based on measurements from 15 fully mature specimens. All sizes are given in millimeters.

Dicrogaster fastigatus n. sp.

Diagnosis: Body conical, spinous, 0.274–0.86 long and 0.14–0.33 thick. Oral sucker spherical, 0.042–0.083 in diameter. Acetabulum spherical, slightly larger than the oral sucker, 0.062–0.094 in diameter. Pre-pharynx present. Pharynx oval to spherical, 0.028–0.052 long and 0.028–0.055 wide. Intestinal crura extend to mid-body region and have terminal expansions.

Testis a single oval structure, situated in the posterior half of the body, measuring 0.073–0.162 long by 0.049–0.097 wide. Seminal vesicle bi-partite, 1 portion within the hermaphroditic sac.



EXPLANATION OF FIGURE

A camera lucida drawing of a lateral view of *Dicrogaster fastigatus* n. sp. scale line = 0.25 mm

Ovary spherical, situated anterior to testis, measuring 0.035–0.087 in diameter. Vitelline gland a single spherical structure, 0.031–0.083 in diameter, situated dorsally between or slightly

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behind crura. Laurer's canal passes from ootype, between crural expansions to dorsal body surface. Uterus with descending and ascending limbs, enters the hermaphroditic sac near its posterior end. Hermaphroditic sac large, extends posteriorly to or beyond acetabulum.

Eggs oval 0.015–0.021 × 0.042–0.052. Adult worms with 20–40 eggs in uterus at one time. Eggs contain well developed miracidia which possess conspicuous eye-spots of oval to triangular shape.

Host: *Mugil cephalus* Linnaeus.
Locality: Grand Isle, Louisiana.
Site: Upper intestinal tract.
Holotype: U. S. N. M. Helm. Coll. No. 38389.
Paratypes: Authors' collections.

DISCUSSION

Present specimens are assigned to the genus *Dicrogaster* on the basis of having the vitelline gland in a single unit. All other genera in the family have 2 or more separate vitelline units. In the genus *Dicrogaster* there are 2 species both of which Looss (1902) described from *Mugil chelo* in European waters. Present material differs from both of the known species in having the vitelline gland in a single lobe instead of in a single bi-lobed unit. A table follows which compares *D. fastigatus* with the other 2 species, namely *D. perpusillus* Looss, 1902, and *D. contractus*, Looss, 1902. It can be seen that *D. fastigatus* is considerably larger than the others.

TABLE I.—*Comparison of the known species of* Dicrogaster/(measurements in millimeters).

	D. fastigatus	*D. perpusillus*	*D. contractus*
Host	*M. cephalus*	*M. chelo*	*M. chelo*
Body length	0.274–0.86	0.3–0.33	0.45
Body width	0.14 –0.33	0.18	0.24
Oral sucker	0.042–0.083	0.066	0.1
Acetabulum	0.062–0.094	0.1	0.125
Pharynx	0.028–0.052	0.018	0.047 × 0.034
Eggs	0.042–0.052 × 0.015–0.021	0.053 × 0.025	0.035–0.04 × 0.023
Miracidial eyespots	Present	Present	Absent

The average length of all specimens measured was 0.677 mm as compared with a maximum reported for *D. perpusillus* of 0.33 mm and a maximum of 0.45 mm for *D. contractus*. The eggs of *D. fastigatus* are about intermediate in size to those of the other species. They average smaller than the eggs of *D. perpusillus* and larger than those of *D. contractus*. Both the oral and ventral suckers of *D. fastigatus* are relatively much smaller than for either of the other species.

REFERENCE

Looss, A. 1902 Die Distomenunterfamilie der Haploporinae. Arch. Parasitol. **6**: 129–143.

RESEARCH NOTE

CERCARIA MEGALURA CORT, 1914, THE LARVA OF A SPECIES OF *PHILOPHTHALMUS*

Cercaria megalura is almost indistinguishable from the larva of *Parorchis* species inhabiting the cloaca of birds, but has been found to develop to maturity in the orbit of those hosts. The chick has been infected experimentally and natural infections have been found in the belted kingfisher and green heron in southern Indiana. *Cercaria megalura* encysts on various surfaces as do *Parorchis* larvae but shows a preference for chitin, thus indicating that natural infections result from eating arthropods. After ingestion, the metacercariae escape from the cysts, migrate up the esophagus to the nasal cavities and develop there for about 4 days in the chick. The still immature worms then pass to the orbit by way of the nasolacrymal canal, become ovigerous by the 14th day, and lay eggs which are ready to hatch by the 35th day or perhaps earlier. The adult is a species of *Philophthalmus*, a genus not previously reported from the United States.—FRANK M. FISHER AND A. FRED WEST, *Department of Biological Sciences, Purdue University, Lafayette, Indiana.*

A NEW SPECIES OF TICK FROM THE TRANS-PECOS REGION OF TEXAS*

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In the course of an ecological survey of the Big Bend region of Texas conducted cooperatively by the Texas A. & M. College, the National Park Service, and the Texas Game and Fish Commission, several specimens of a new species of tick belonging to the genus *Aponomma* were collected by William G. Degenhardt. These collections pose some interesting problems since this is the first record of this genus from Continental North America. The possibility of an importation appears to be improbable since the host, the Trans-Pecos Rat Snake, is native to the rather remote area where the collections were made.

Aponomma elaphensis, n. sp.

Holotype male and *allotype* female, from Chisos Mountains, 3700 feet elevation, Big Bend National Park, Texas, Summer, 1956, W. G. Degenhardt, in U. S. National Museum. Host: *Elaphe subocularis* Brown, Trans-Pecos Rat Snake.

Paratypes: 18 males, 24 females, 48 nymphs, data as above, deposited in U. S. National Museum; Rocky Moutain Laboratory; University of Maryland; Dr. J. Bequaert, University of Houston; Texas State Department of Health Laboratories; University of California; Division of Animal Health and Production, C.S.I.R.O., Veterinary Parasitology Laboratory, Yeerongpilly, Queensland, Australia; Division of Veterinary Services, Department of Agriculture, Onderstepoort, Union of South Africa; Illinois Natural History Survey Collection; and Texas A. & M. College, Department of Entomology Collection.

Description of male. (All measurements are for the holotype, and are in millimeters.)

Suboval, widest about first festoon; length, apex of scapula to posterior margin of body, 2.68; width, 2.38. Color pale yellowish brown.

Capitulum. Length, tip of palpus to posterolateral corner of basis capituli, .63; width of basis capituli, .49; basis capituli subtriangular, posterior margin nearly straight, dorsal surface with a few very fine inconspicuous punctations; palpi moderately long, segment II not twice as long as segment III.

Hypostome. Shape as figured; moderately long, about .39; dentition 2/2; teeth occupy about 67% of length.

Scutum. Pale yellowish brown; inornate; slightly narrowed anteriorly; a few very fine inconspicuous punctations present peripherally and in scapular region; without elevations, depressions, hairs or lateral grooves; festoons short, wider than long.

Legs. Moderate in length and size. Coxae I short, rounded knoblike at posteromedial angle. Moderately long and acute external spurs on all coxae. Length of tarsus I, .51; metatarsus, .22. Length of tarsus IV, .33; metatarsus, .19. All tarsal claws long and slender.

Spiracular plate. Long and narrow with the longer axis longitudinal as figured.

Genital aperture. Situated at the level of the intervals between coxae II and III.

Ventral surface. The area bounded anteriorly by basis capituli, laterally by the legs and posteriorly by the anal opening, hyaline with very fine closely spaced transverse striations extending to lateral margins. Remainder of ventral surface, except legs, light yellowish brown with numerous moderate-sized punctations. Genital grooves slightly divergent to juncture of anal groove, thence widely so until they fade out on each side posterior to the stigmal plate. Paired lateral folds arise at a transverse fold anterior to genital aperture at the level of coxae II and extend posteriorly between genital grooves and coxae to coxae IV, become widely divergent and fade out on each side at the first festoon, posterior to spiracular plates.

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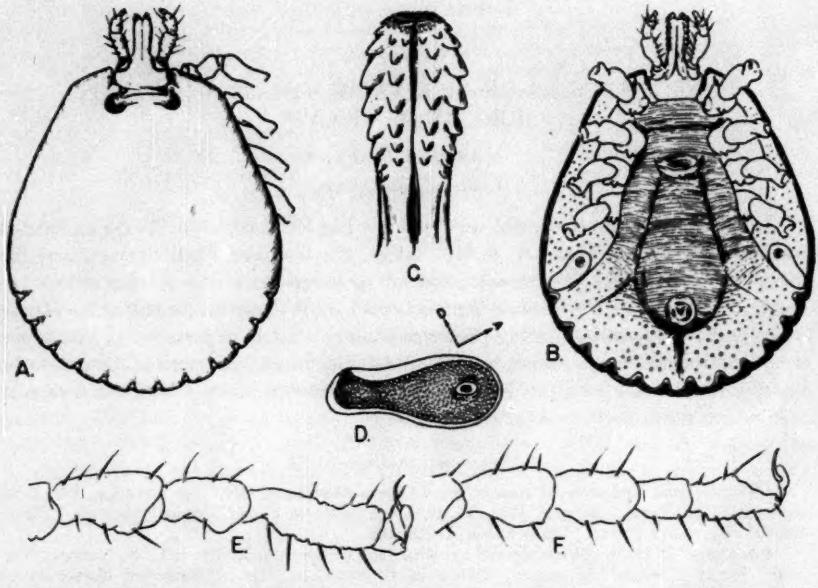

FIGURE 1. *Aponomma elaphensis* n. sp. Male.
A. Capitulum and scutum, dorsum. B. Capitulum and coxae, venter. C. Hypostome. D. Spiracular plate (A = anterior; D = dorsal). E. Metatarsus and tarsus, leg I. F. Metatarsus and tarsus, leg IV.

Description of female. (All measurements are for the unengorged allotype.)

Suboval, widest at about first festoon, without marginal grooves. Length, apex of scapula to posterior margin of body, 3.05; width, 2.79. Color pale yellowish brown. The largest engorged female measured 14.2 in length, from tip of scapula to posterior margin, and 5.4 in width.

Capitulum. Length, apex of palpus to posterolateral corner of basis capituli, .72; width, .63; basis capituli subtriangular; posterior margin nearly straight. Porose areas rounded, moderate in size, slightly depressed and inconspicuous. Palpi moderately long, segment II not twice as long as segment III.

Hypostome. Shape as figured. Moderately long, about .41; dentition 2/2; teeth occupy about 76% of length.

Scutum. Length, 1.36; width, 1.87. Posterolateral margin straight; posterior margin broadly rounded. Cervical grooves wide and shallow, extending almost to the posterolateral margins. Inornate, pale yellowish brown, punctations small, sparse and inconspicuous.

Legs. Moderate in length and size, coxae I short, rounded and knoblike at medial angle. Moderately long and acute external spurs on all coxae. Length of tarsus I, .62; metatarsus, .30; length of tarsus IV, .40; metatarsus, .25.

Spiracular plate. Shape as figured with longer axis longitudinal.

Genital aperture. Semicircular and situated between coxae III.

Ventral surface. With fine closely spaced striations; impunctate and beset with numerous small short white inconspicuous setae. Genital grooves slightly divergent to juncture with anal groove thence widely divergent until they fade out on each side posterior to the stigmal plate. Paired lateral folds arise at a transverse fold anterior to genital aperture at the level of coxae II and extend posteriorly between genital grooves and coxae to coxae IV, become widely divergent and fade out on each side at the first festoon posterior to spiracular plates.

Description of nymph. (Unengorged)

Length, tip of scapula to posterior margin of body, 1.63; width, 1.48. Broadly oval. Hyaline with inconspicuous transverse striations.

Capitulum. Length, tip of palpus to posterolateral corner of basis capituli, .42; width of basis capituli, .35. Shape as described for female.

Hypostome. Shape as figured. Length, .23; dentition 2/2.

Scutum. Length, .38; width, .96. Weakly sclerotized, otherwise similar to female.

Legs. Similar to female. Length of tarsus I, .37; metatarsus, .13. Length of tarsus IV, .23; metatarsus, 13.

Ventral surface. Same characters as female.

According to Schulze's (1936) and Roberts' (1953) descriptions this species resembles the Australian species *Aponomma auruginans* Schulze in being inornate and having a hypostome dentition of 2/2 but differs in several respects. *A. elaphensis* is smaller, has only 1 spur on coxae I and the tarsi are not strongly humped. The posterior margin of the basis capituli is straight and not concave as it is in the Australian species.

The host relationships of *A. elaphensis* and *A. auruginans* appear to be quite different since the latter has been taken only from the wombat. *A. elaphensis* has been collected only from snakes.

Mr. William G. Degenhardt is preparing a note on the ecology and host associations of *A. elaphensis.*

The writer wishes to express his sincere appreciation to Mr. Glen M. Kohls for his critical review and many helpful suggestions during the preparation of the manuscript. Acknowledgment is also due Mr. William G. Degenhardt for collecting the ticks.

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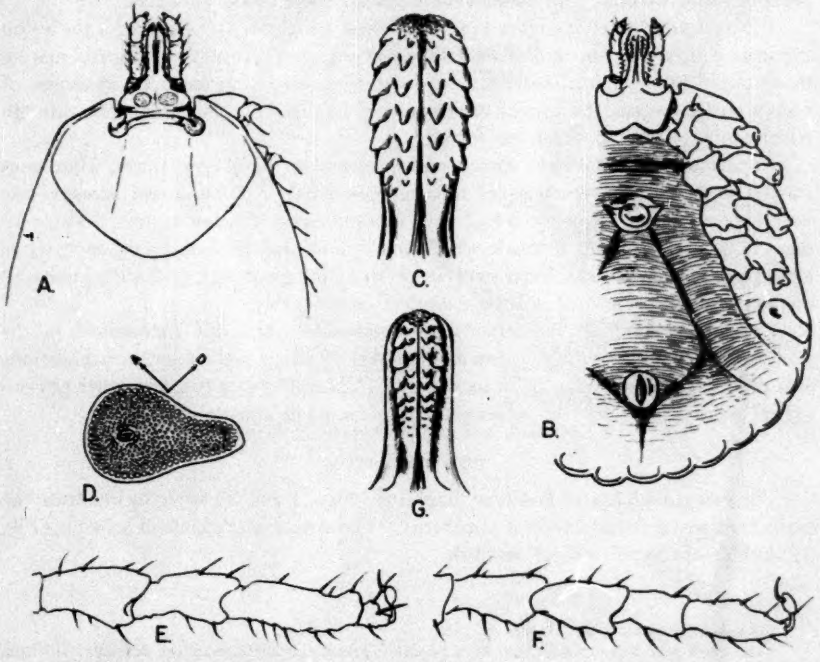

FIGURE 2. *Aponomma elaphensis* n. sp. Female.

A. Capitulum and scutum, dorsum. B. Capitulum and coxae, venter. C. Hypostome. D. Spiracular plate (A = anterior; D = dorsal). E. Metatarsus and tarsus, leg I. F. Metatarsus and tarsus, leg IV. G. Hypostome, nymph.

A PERFUSION APPARATUS FOR MAINTENANCE AND OBSERVATION OF SCHISTOSOMES IN VITRO

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A rising interest in techniques for maintenance in vitro of many species of endoparasites has produced reports of successful cultivation of certain intestinal nematodes (Weinstein, 1953, 1956), partial development of liver trematodes (Yokagawa et al, 1955) and of human blood flukes (Senft and Weller, 1956). In the field of schistosome research, a number of attempts at prolonged maintenance in a specially constructed apparatus have been reported (Newsome and Robinson, 1954).

It appeared that development of techniques whereby adult helminths could be watched and photographed under conditions closely simulating the natural host environment would be helpful.

In the case of the schistosomes, exact definition of nutritional requirements for growth and reproduction has yet to be realized, even though the worms can be induced to grow, develop, and mate in various salt-serum media. In addition, little has yet been done to assess the significance of the physical aspects of a blood stream environment. Among these would be the effect of dissolved gases, pressure, temperature, flow of fluid, and osmotic tensions on these blood parasites.

We have addressed ourselves to some of these problems by designing a perfusion apparatus, in which the flukes may be observed under conditions approximating those found in the portal system. In order to provide the basic environment of such a portal system, the worms were confined in shallow plastic channels, through which sterile, warm medium was forced.

Experiments to date have shown no improvement in survival times, when contrasted to the test-tube techniques reported previously. *Schistosoma mansoni* has been kept alive and active for 6 to 7 days in a pulsating, flowing system, and for 16 days in a static system, through which fresh fluid was flushed every day. It is anticipated that with additional experience with the apparatus, and with improved media, periods of survival will be extended considerably.

Preliminary findings indicate that schistosomes which are maintained in the perfusion apparatus require maintenance fluids of much higher serum proportions that those kept in test tubes in an incubator. Data concerning this and other physiological observations will be presented in a subsequent communication.

THE APPARATUS

The general apparatus and flow diagrams (Figs. 1 and 2) serve to illustrate the main features of the mechanical apparatus. The temperature control scheme (Fig. 3) outlines the servo-control network.

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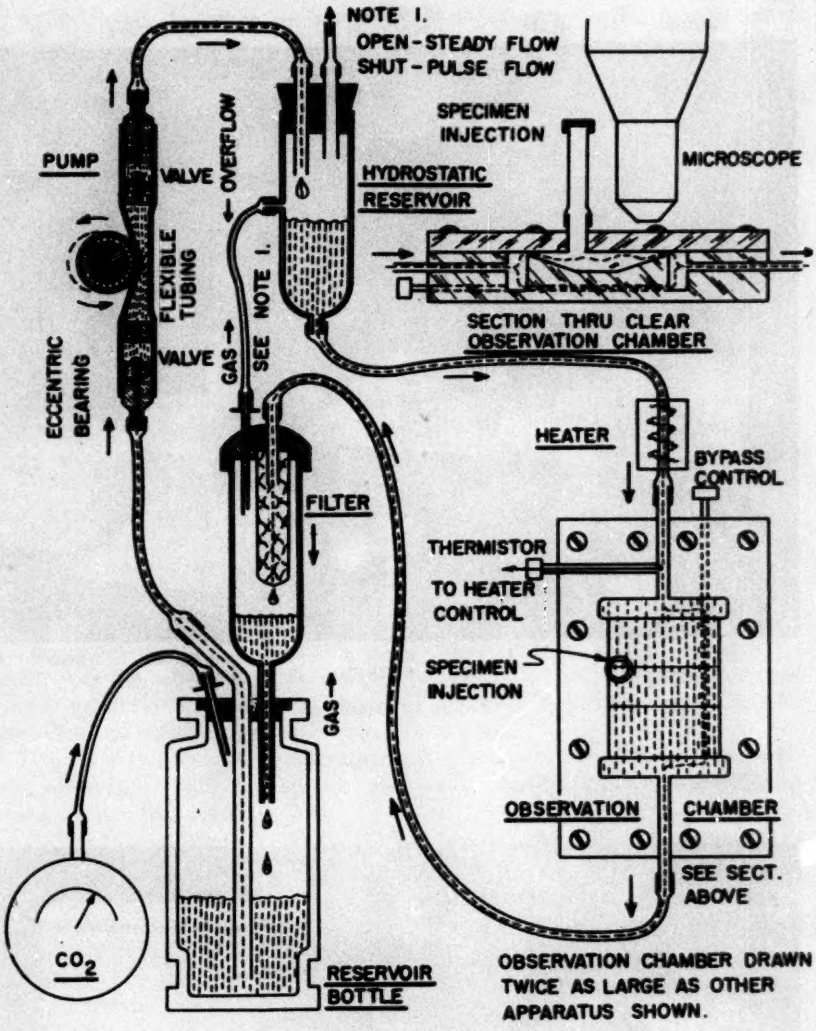

FIGURE 1. Apparatus and flow diagram.

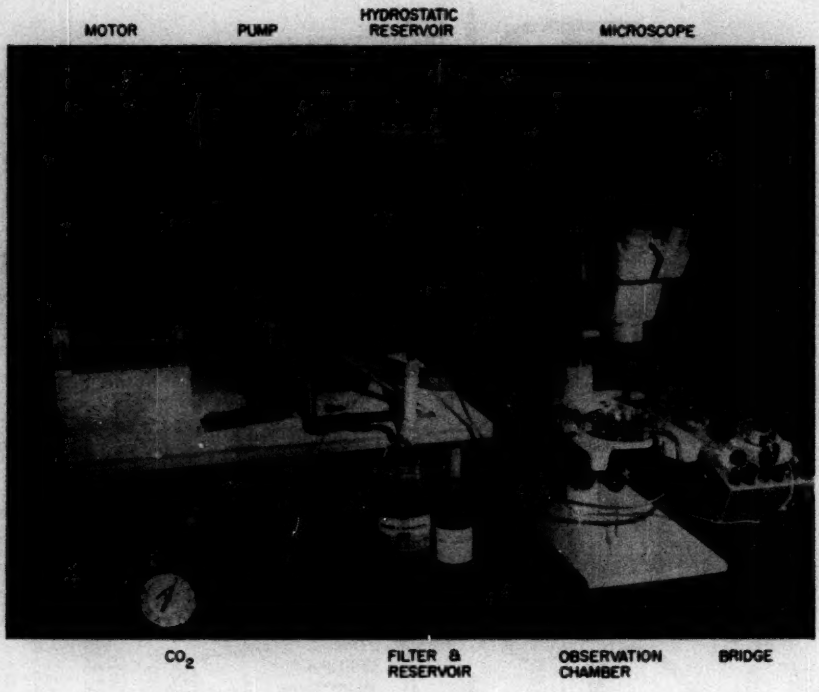

FIGURE 2. Apparatus.

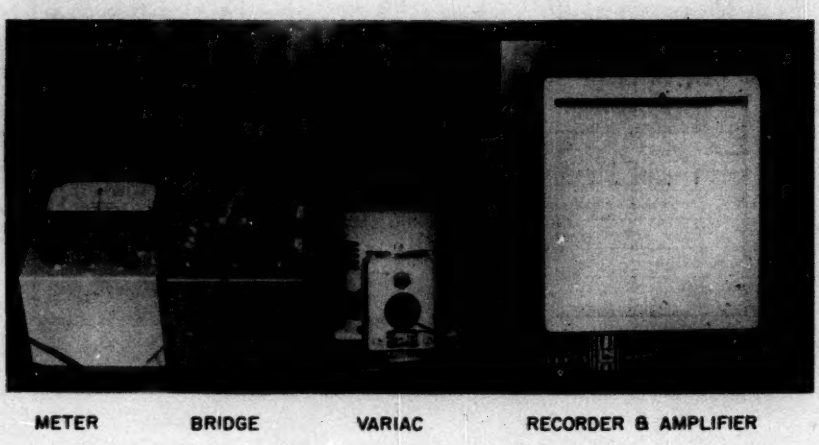

FIGURE 3. Temperature control and recording equipment.

a. *The Pump.* Pulsatile flow is achieved by utilizing an eccentric bearing-device which alternately squeezes and releases vinyl plastic ("Tygon") tubing, forcing fluid past 2 check valves. These valves are made of stainless steel, while the valve seats are polytrifluoro-chlorethylene ("Teflon"). The eccentric bearing is driven by means of a standard laboratory stirring motor. A cone drive and idler wheel assembly provide variations in pulse rate from about 48 to 300 per minute. The stroke-volume output is altered by changing the impinging pressure of the eccentric on the plastic tubing. The machine is generally set to deliver 7–10 ml per minute at about 80 pulses per minute. Flow rates are estimated by counting the number of drops per minute which fall into the reservoir bottle. For accuracy, an initial calibration of output is required.

The pressure in the observation chamber can be set as desired by adjusting the height of the hydrostatic reservoir. The usual position provides 8–10 cm H_2O static pressure and 1–2 cm H_2O dynamic variation between "systolic" and "diastolic" readings. These values were derived by actual measurement during 1 experiment, but are not necessarily identical from time to time. They are believed to approximate the portal-pressure readings of the normal male as measured in other studies (Baylor, personal communication).

b. *The Hydrostatic Reservoir.* It has proved desirable to be able to change at will from a pulsatile to a gravity flow in the observation chamber. To allow such changes, a hydrostatic reservoir has been interposed between the pump and the chamber. Since adjustment of the reservoir height changes the gravity flow rate, proper manipulation of this height allows the gravity flow to match the output of the pump almost exactly. The overflow vent diverts any excess fluid into the reservoir bottle. A cotton-stoppered vent at the top of the hydrostatic reservoir assures gravity flow; it also provides egress for CO_2-O_2 mixtures which may be introduced via the reservoir bottle from time to time.

The closing of both the overflow vent and the air vent of the hydrostatic reservoir converts the flow through the observation chamber from a non-pulsating gravity stream to a pulsating flow.

c. *Temperature Control* (*Fig. 3*). Fluid passes through a 10-cm length of stainless steel tubing before entering the observation chamber. A length of nichrome wire of about 300 ohms resistance which is wrapped around the metal tubing is heated by the servo-controlled AC current. The fluid is warmed in passage through the tubing; it next passes by a thermistor (Veco 31A1, Victory Engineering Company), the resistance of which varies inversely with ambient temperature. The thermistor element is used as 1 arm of a precision Wheatstone bridge. A shift in the resistance of the thermistor upsets the balance of the bridge, and any change in bridge voltage is detected and continuously recorded by the amplifier (Leeds-Northrup Speedomax H). This recorder was so adapted that movement of the indicating pointer causes a change in the output voltage of a variable transformer which energizes the heater. Continuous and proportional heat control, without recourse to a large volume of fluid or water bath, is thus provided within a tolerance of $\pm 0.1°$ C.

Although most observations are made at 37° C, the bridge settings may be adjusted to provide other desired operating temperatures. It will be noted that the main volume of the perfusion fluid remains at or near room temperature; that por-

tion passing through the chamber is the only amount heated to operating temperature at any given time.

d. *The Chamber* (*Fig. 4*). A thick block of acrylic-methacrylate plastic ("Lu-

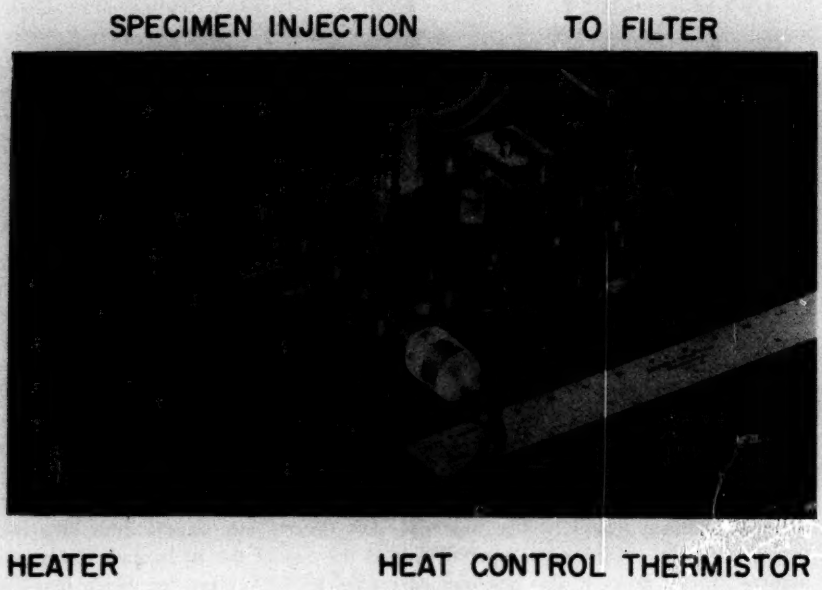

FIGURE 4. Observation chamber.

cite") $9 \times 6 \times 1.25$ cm was milled so as to provide a shallow, sloped trough in which to confine the schistosomes. The trough is about 0.2 cm deep at its center, and provides about 0.012 cm clearance under the chamber cover at each end. Because of this sloping configuration, flukes varying considerably in size may be confined within the chamber, while fluid passes through relatively easily. The worms are able to move freely in the deepest part of the chamber, but eventually they are swept downstream to a point where they are confined by the narrowness of the chamber.

In some earlier models, tapered grooves, instead of a sloped chamber, were provided. The presumption was that worm pairs would migrate down these channels much as they do in the portal system. However, the worms did not survive for more than 24–36 hours in such models, possibly because of inadequate circulation past them, or because of pressure damage. Thus far, results with grooved channels have been most disappointing.

A by-pass control has been found to be convenient. This helps to adjust the flow rate in the sloped chamber, and also serves as an exit for CO_2 bubbles which form when previously chilled perfusion fluids are brought to operating temperature too quickly.

Schistosomes are introduced into the chamber by means of a sterile Pasteur pipette. The system is first brought to operating temperature. Then the chamber

is isolated from flow by occluding the inflow and outflow tubing with screw clamps. The worm is then quickly introduced into the chamber via the specimen-injection tubing. As fluid flow is re-established, the worm is swept into a position where it is held fast and can be observed.

Entrapped bubbles in the observation chamber may be released by maneuvering them under the specimen-injection tubing and then venting them to air by momentarily loosening the cap.

e. *The Reservoir.* A 250-ml graduated bottle stoppered with a rubber diaphragm cap serves as the main reservoir. A commercial filter (Abbott Blood Recipient Unit 4447) commonly used for blood transfusions, provides the necessary plastic tubing and a Monel-metal filter. This filter is used as a grid for the oxygenation of the fluid and added Red Blood Cells. O_2-CO_2 mixtures are introduced by means of a cotton-stoppered tube and needle which is set into the diaphragm cap of the reservoir bottle. Excess gas is vented out through the overflow tubing and the hydrostatic reservoir. Gas pressure may be used to fill the pump when the system is started into operation, or when a fresh bottle of medium is used.

STERILIZATION OF EQUIPMENT

The maintenance of axenic conditions during prolonged operation has proved to be a formidable task, particularly since most of the components of the perfusion apparatus cannot be steam-sterilized. Two substitutes for steam-sterilization have been used in this laboratory and are described below. The first relies on careful aseptic techniques during assembly of the apparatus. The alternate method employs a gas-penetration sterilizing procedure which requires a pressure-vacuum chamber and a special gas mixture. It is, however, far more reliable as a method for sterilizing the equipment.

In the first method, the following procedures are used:

1. Except for tubing, all plastic or stainless steel parts are scrubbed with a detergent (Alconox), and then repeatedly rinsed in sterile distilled water.

2. The apparatus is oven-dried at 55° C and then assembled in an ultraviolet light equipped bacteriological hood. Sterilized silicone stopcock grease (Dow-Corning) is used as a waterproofing agent on all threaded joints and at the plastic interfaces of the observation chamber.

3. When beginning operation, sterile Hanks' solution, containing no antibiotics, is flushed through the apparatus and discarded. Precautionary sterility tests, using blood agar plates and thioglycollate broth tubes, are made of aliquots of this discarded fluid.

4. Commercially available sterile plastic equipment is used to provide lengths of tubing and the needle assemblies.

5. Antibiotics are added to the circulating medium; penicillin at 100 units per ml final concentration, together with streptomycin at 100 micrograms per ml final concentration, or alternatively tetracycline-oleandomycin (Signemycin, Pfizer) at 10 micrograms per ml final concentration have been tried. The latter antibiotic appears to work satisfactorily.

Using the gas-penetration technique, the assembled perfusion apparatus can be sterilized in a mixture of ethylene oxide and Freon gases. The apparatus is first washed and dried and then fully assembled, except for the addition of the reservoir bottle containing the perfusion mixture. Needles, which are later to be inserted

into the reservoir bottle diaphragm, are protected with cotton-stoppered plastic covers during sterilization.

The unit is placed in a small vacuum-pressure vessel from which the air is exhausted to 28 inches vacuum. Ethylene oxide mixture (11% ethylene oxide, 48.5% Freon-11 and 48.5% Freon-12),* enough to raise the pressure to 8 pounds, is then quickly introduced. This pressure is maintained for 4–5 hours at room temperature (70° F). The chamber is then again evacuated and the gas is discharged through a chemical hood. The unit may then be removed, aired for about ½ hour, and then set up on the pumping stand for operation.

Sterilization with ethylene oxide, using different concentrations and various vehicle gases, has been comprehensively studied by Phillips and Kaye (1949) and by Phillips (1949). The adaptation for use in laboratories doing in vitro work while using plastic and other non-heat-sterilizable materials is obvious. Tests in this laboratory have shown that contaminated units, from which previously *Escherichia coli, Aerobacter aerogenes* and a fungus were grown, were sterilized by the gas-sterilization technique described above. In addition, aliquots of perfusion fluid which were taken as late as 18 days after the beginning operation of the perfusion machine have remained sterile when incubated on blood agar or in thioglycollate broth. No steps were taken to remove or neutralize any added antibiotics from such aliquots.

SUMMARY

A detailed outline for construction, sterilization, and operation of a "heart-lung" apparatus for maintenance of schistosomes in vitro is given. By means of this device, schistosomes may be observed continuously for periods of several days to a week in an environment which simulates the portal system. Provision has been made for altering the physical conditions of temperature, pressure, and flow rates. The machine can be set to provide pulsatile or gravity flow at will. Composition of the perfusion fluids may be changed so as to assess the effect on worms under study.

ACKNOWLEDGMENTS

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* Available on special order from Olin Mathiesen Co., Rutherford, N. J.

CHEMOTHERAPY OF EXPERIMENTAL SCHISTOSOMIASIS. I. DRUG ACTIVITY AND MODE OF ACTION OF A NEW THIOXANTHONE DERIVATIVE

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In addition to the antimonials, only a few thioxanthone derivatives have been employed in the chemotherapy of schistosomiasis. Nevertheless, the results obtained with the drugs now available are relatively poor and there exists a great need for new schistosomicidal agents. Raison and Standen (1954, 1955) have recently reported marked schistosomicidal activity of symmetrical diaminodiphenoxyalkanes. Caldwell and Standen (1956) and Hill (1956) demonstrated that p-aminophenoxyalkane derivatives and some compounds of the homologous series of diphenoxyalkanes are also active against *S. mansoni*. Ring substituents of diphenoxyalkanes are less effective (Standen and Walls, 1956). Some rhodanines, hydantoins and N-(9-Xanthenyl) amides have been demonstrated by Luttermoser and Bond (1954) and Bond and Luttermoser (1954) to have therapeutic activity.

The results obtained in mice experimentally infected with *S. mansoni* and treated with a new thioxanthone derivative are reported in this paper, along with some observations concerning the mode of drug action.

MATERIALS AND METHODS

The drug used was synthesized by "Ciba" laboratories and received the number 17'581. Chemically it is 1-(diethylaminoethylamino)-4,6,8-trimethyl-5-azo-thioxanthone-hydrochloride. This yellowish compound is soluble to 5% in water and the solution is stable at room temperature when protected against light. The LD_{50}, for mice, by the intravenous route, is 60 mg/kg. When given orally, in the relatively large dose of 2 gm/kg, it produces only slight toxic symptoms. In preliminary tests performed in the Ciba Laboratories, the drug revealed therapeutic activity against *S. mansoni* infection.

Assessment of drug activity: Albino mice, weighing 18 to 20 g, were inoculated intraperitoneally with 200 cercariae derived from naturally infected *Australorbis glabratus*. The treated mice received 300 mg/kg of drug, orally, once a day for 5 days in two series starting on the 32nd and 42nd days of infection. The animals were sacrificed 52 days after inoculation. The assessment of drug activity was based on the determination of the number of granulomas isolated from the liver of the treated and control animals (Pellegrino and Brener, 1957; Brener, Pellegrino and Oliveira, 1957) and on the number of living worms recovered after perfusion of the liver and mesenteric veins.

Mode of drug action: Mice were treated with drug 17'581 using the same dosage as given above, in a single series starting on the 50th day of infection. The treated mice were sacrificed at different periods and worms were collected separately from the liver and mesenteric portal veins. For comparative studies a group of 50 mice were treated by Fuadin (125 mg/kg, twice a day, for 5 days) intraperitoneally.

Dead or immobilized worms, easily recognized in the liver by press preparations, were freed from the inflammatory tissue for further study. The development of tissue reaction around the worms was followed histologically.

RESULTS

The results obtained with the drug 17'581 are given in Table 1. No living schistosomes were recovered in treated mice and only a small number of granulomas were isolated in the treated group. Graph I compares the worm shift to the liver after feeding drug 17'581 and Fuadin administration.

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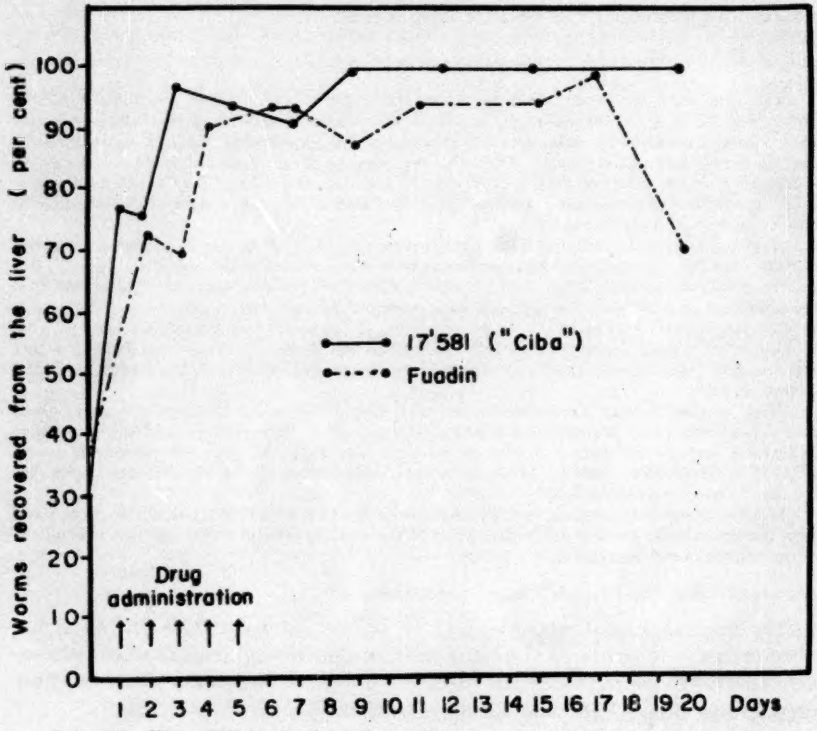
TABLE I.—*Number of granulomas and living worms in mice treated by 17'581.*

Animal number	Treated animals		Controls	
	Worms	Granulomas	Worms	Granulomas
1	0	0	8	100
2	0	828	66	4404
3	0	0	24	1032
4	0	0	75	4476
5	0	0	70	1455
6	0	0	0	0
7	0	381	120	2577
8	0	300	16	120
9	0	414	85	4000
10	0	424	55	2476
11	0	0		
12	0	0		
Mean	0	195.5	51.9	2054.0

DISCUSSION

In addition to the discovery of new schistosomicidal agents there is also a great interest concerning the mode of action of active drugs. Except for the effects on the worms' reproductive organs, with inhibition of mitoses, little is known about macroscopic and functional disturbances produced by the drugs. Bang and Hairston (1946) demonstrated the shift to the liver and loss of digestive tract pigment by the worms during antimonial therapy. Kikuth and Gonnert (1948) also observed, with Miracil, migration to the liver, depigmentation, decrease in size and increased



GRAPH I.—*Worm shift to the liver observed in mice treated with Fuadin and 17'581.*

fragility of the worms. Standen (1953, 1955) in detailed studies, using Fuadin, Nilodin and a diphenoxyalkane found that the worms lose muscular tone, are rendered unable to hold on to the vessels and are carried passively to the liver; they also cease to feed, become smaller and depigmented. Separation of sexes is particularly evident after diphenoxyalkane therapy. The worms retained in the liver be-

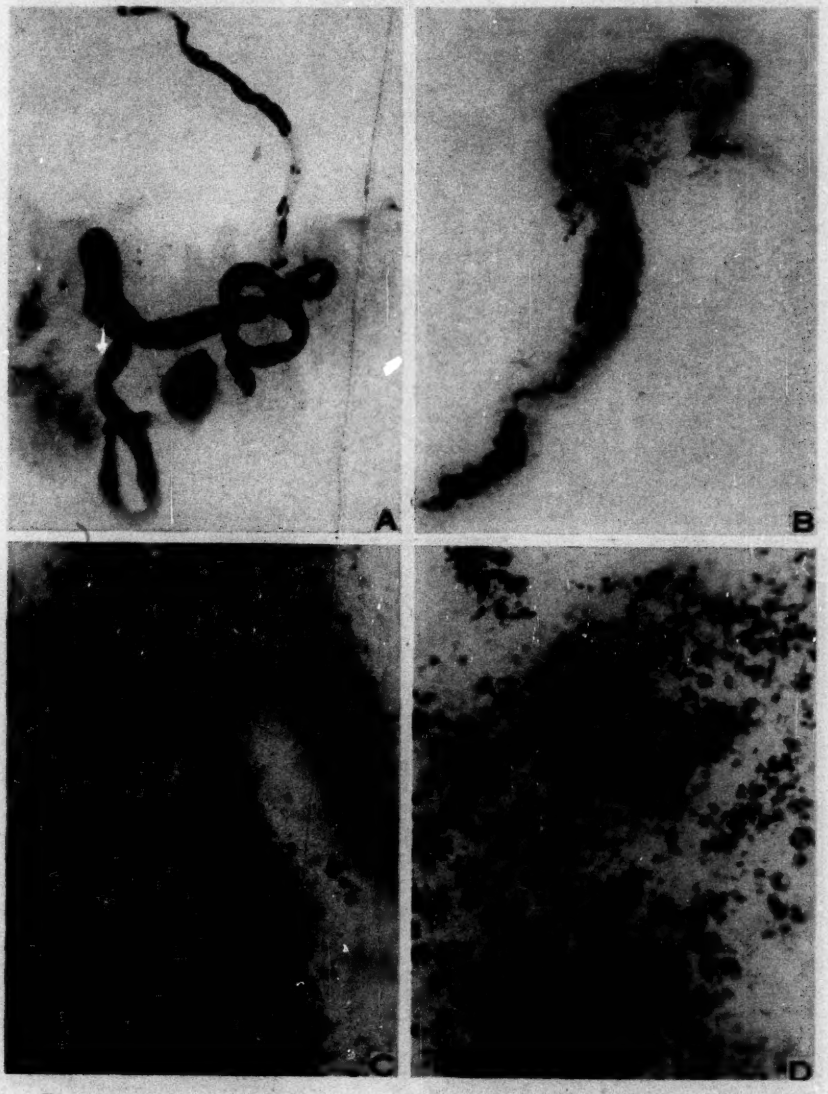


FIGURE 1. *S. mansoni* in the liver of treated animals (press preparations): A, Living worm partially entangled by inflammatory tissue. Histological section of the same worm is seen in Figure 2A. B, Worm partially disintegrated. C, Advanced stage of worm disintegration. D, Residual pigment of a completely destroyed worm.

come subject to a gradual disintegration by phagocytic action which may begin when the worms are still living. A similar sequence was observed by Raison and Standen (1955) with the diaminodiphenoxyalkanes. Gonnert (1955), using Nilodin, con-

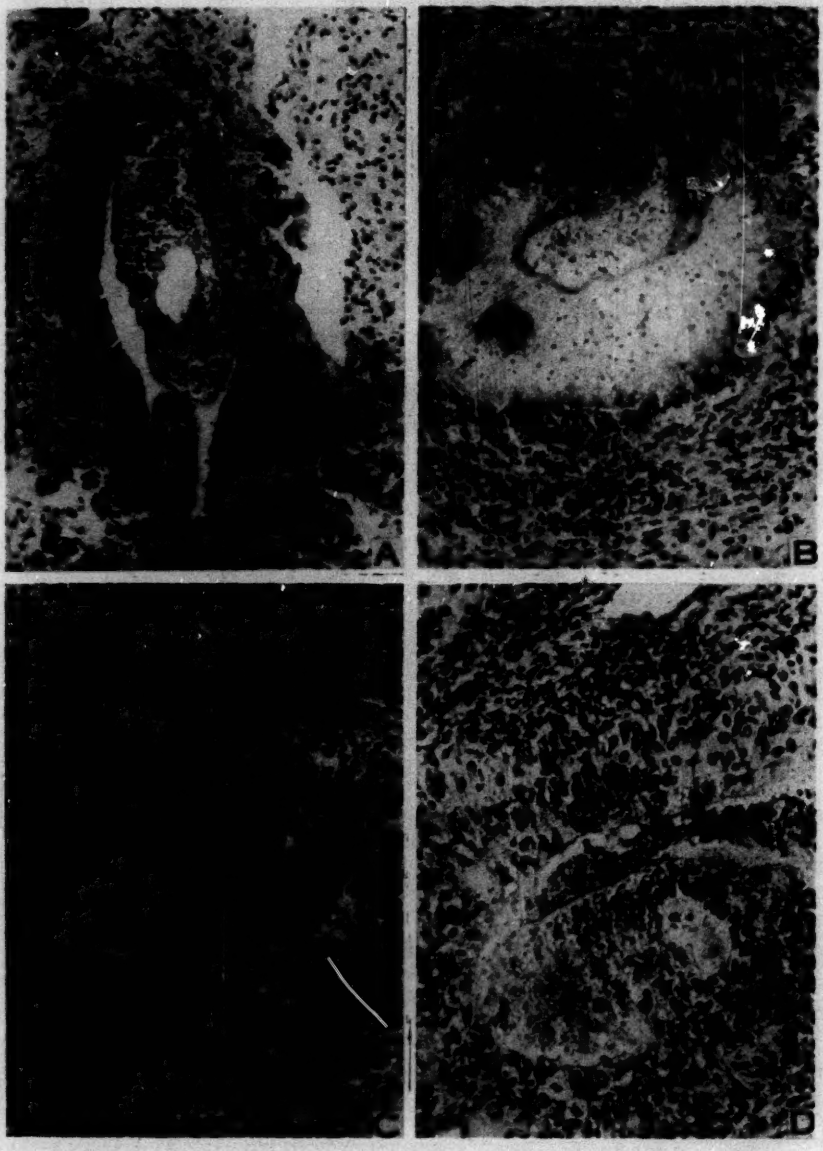


FIGURE 2. *S. mansoni* in the liver of treated animals: A, Section showing partial adherence of the worm to the vessel wall. B, C, D, Different stages of phagocytic action and worm disintegration.

firmed the findings reported by the above-mentioned authors and described vacuolization of the worm cuticle as the first degenerative sign.

When mice are treated with drug 17′581 the shift of worms to the liver is evident 24 hours after the beginning of treatment. The rate of migration is similar to that found with Fuadin (Graph I). In this respect the drug 17′581 seems to differ from Nilodin whose action is fully evident only some days after commencement of therapy (Standen, 1953). From the 3rd day, an increasing number of worms are trapped in the liver and cannot be removed by perfusion. These worms, however, are easily extricated by dissection, probably because at this stage the worms are retained by only slight adhesions. After a week, the worms are completely ensheathed and entangled by inflammatory tissue. The phagocytic process begins at the point where the worm cuticle is in contact with the vessel wall (Fig. 2, A). The immobilization of the worm by the inflammatory tissue may occur while the worm is still alive (Fig. 1, A; Fig. 2, A). From the 12th day on, all the worms are in the liver. Most are surrounded and invaded by inflammatory cells (Fig. 2, B). The chief components of this inflammatory reaction are eosinophiles, neutrophiles, and macrophages. In contrast to the other schistosomicidal drugs already described, drug 17′581 causes little loss of pigment (Fig. 1; Fig. 2, C). The depigmentation of schistosomes after effective treatment is believed to be related to the decrease of muscular tone which produces disgorgement of pigment and impairment of feeding (Standen, 1953; 1955). With the drug 17′581 the persistence of gut-pigment was probably due to the rapid action of this compound. Nevertheless, other signs indicating muscular disturbances, such as hepatic shift, separation of sexes and decrease of active movements in vitro were observed.

The data presented strongly suggest that the now available schistosomicidal agents act by a similar mechanism producing functional disturbances followed by an non-specific phagocytic destruction of worms in the liver. The difference in the activity of drugs, as claimed by Standen (1955), lies in the "relative potential for immobilizing the worms."

SUMMARY

A new thioxanthone derivative, 1-(diethylaminoethylamino)-4,6,8-trimethyl-5-azo-thioxanthone-hydrochloride, with tested for therapeutic activity in mice experimentally infected with *Schistosoma mansoni*. Significant decrease in the number of granulomas and death of the worms was observed in the treated animals. The study of the mode of action of this thioxanthone showed that an early migration of the worms to the liver occurred after drug administration. This shift was followed by immobilization of the worms by inflammatory reaction which produced disintegration of the schistosomes.

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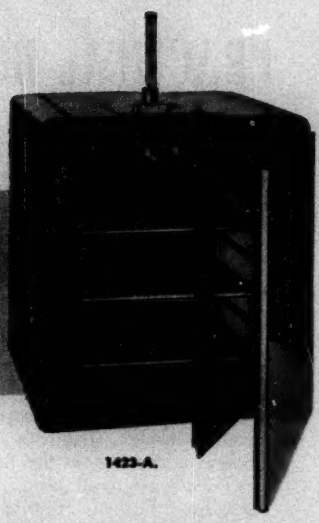

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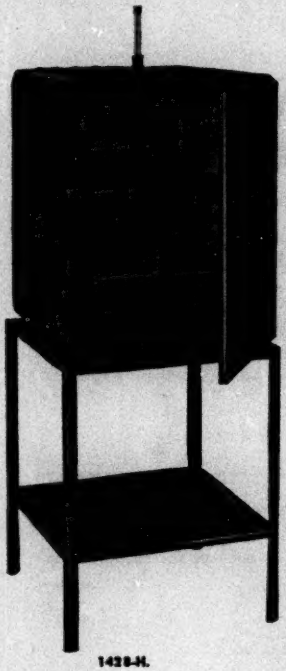

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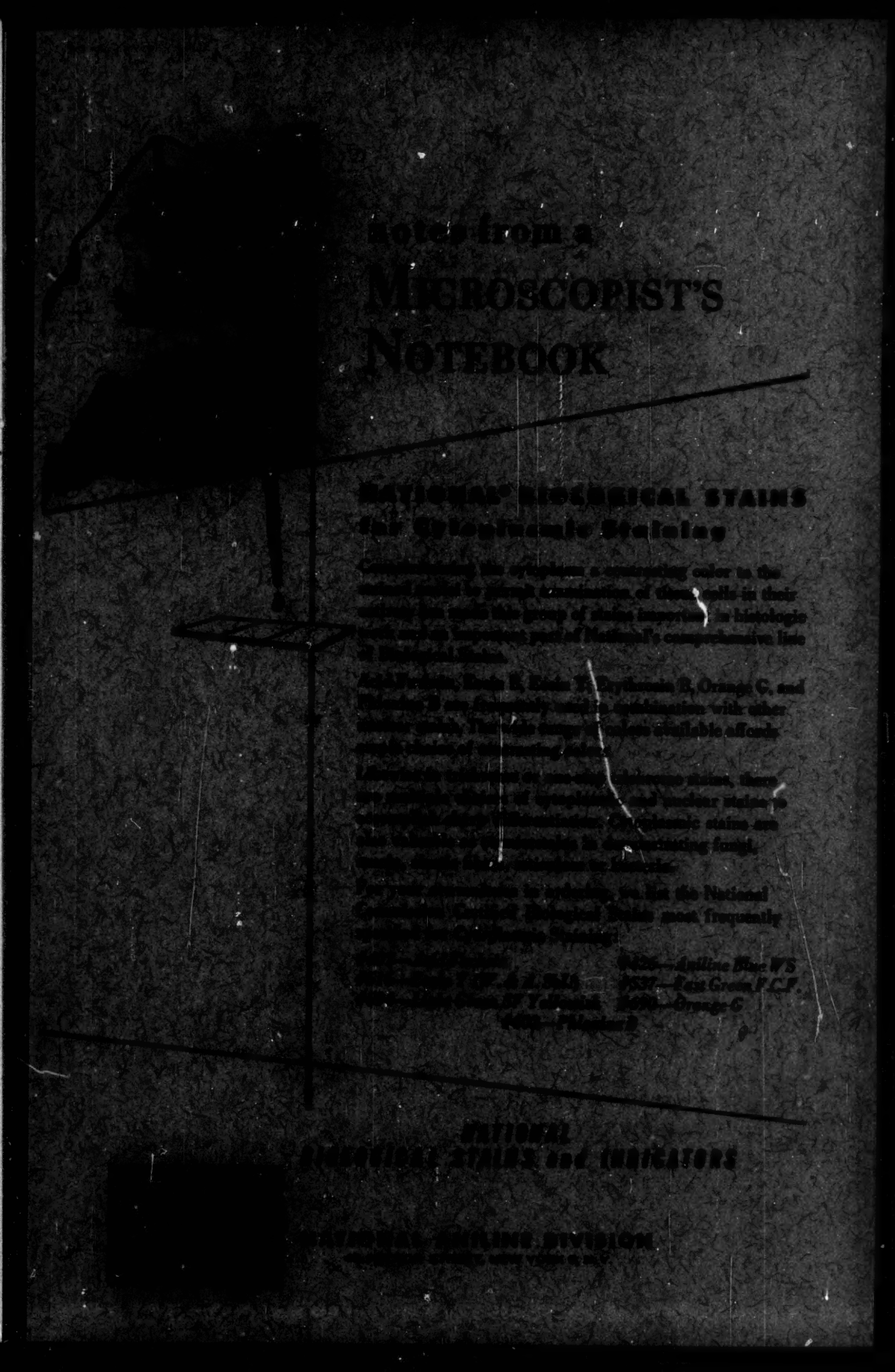

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